INVESTIGATING THE RELATIONSHIP BETWEEN PRO-APOPTOTIC, ANTI-APOPTOTIC AND PROLIFERATION PATHWAYS MEDIATED BY TNF-RELATED APOPTOSIS-INDUCING LIGAND (TRAIL)

A

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Investigating the relationship between pro-apoptotic, anti-apoptotic and proliferation pathways mediated by TNF-related apoptosis-inducing ligand (TRAIL)

1. Abstract:

Triple Negative Breast Cancer (TNBC) being devoid of the hormonal receptors manifests a difficult challenge for different therapeutic approaches. As TRAIL (TNF Related Apoptosis Inducing Ligand) is known for its ability to cause damage specifically to the tumor cells. Whole transcriptome analysis of MDA-MB-231 cell line treated with rhTRAIL showed upregulation of more than 1.5fold in nine genes from the complement pathway. Kaplan-Meiers plotter showed Complement component 4B (CFB) to be positively corelated with relapse free survival in breast cancer patients. CFB was also observed to be co-related and co-expressed with TRAIL in TNBC patients. Because of the heterogenicity of breast tumor population, some cells are inherently resistant to TRAIL induced apoptosis. Via microarray analysis of the selected resistant cells, we observed CDH1 to be downregulated in the resistant cell population in comparison to the sensitive cells. Upregulation of CDH1 sensitized the resistant cells towards rhTRAIL mediated cell death. Another commonly used approach to increase the effectiveness of the TRAIL treatment is through combination therapy. Theophylline has a cytotoxic effect on the MDA-MB-231 cell line. It increased ROS production causing DNA damage and lipid peroxidation. We further observed theophylline to increase the level of TNFR1, thereby activating the caspase mediated apoptotic pathway. Moreover, rhTRAIL in combination with theophylline caused increased cell death in comparison to rhTRAIL or theophylline alone. Combination treatment increased the levels of DR5 in the rhTRAIL and theophylline treated cells. Therefore, we can conclude that CFB and CDH1 can be used as biomarkers for TRAIL therapy in breast cancer. Furthermore, rhTRAIL in combination with theophylline could be a possible alternative for breast cancer therapy.

2. Introduction:

Breast cancer is the most potent nemesis of women's health worldwide. Every year as per World Health Organization (WHO) 2.1 million new breast cancer cases are registered. In 2018 around 627,000 women lost their lives because of breast cancer, which comprises 15% of the total death in women related to all cancer types (WHO) [1]. In recent times the rate of breast cancer has

significantly increased in south-eastern Asia, including India. In the present era a heterogenous disease like breast cancer is difficult to combat as it comprises of different sub-types such as estrogen receptor (ER) or progesterone receptor (PR) positive which includes 60-70% of all breast cancer cases, human epidermal growth factor receptor 2 (HER2) protein amplified or modified consist of 15-30% and 10-15% cases comprises of lack of ER and PR expression and HER2 upregulation regarded as Triple Negative Breast Cancer (TNBC)[2]. In the absence of all three receptors, TNBC patients cannot be treated with receptor targeted therapy or any other endocrine treatments[3]. TNBC comprises 15-20% of all breast cancer patients, it's more common in lower age group patients and is more prone to early relapse and poor diagnosis[4]. Due to the chemotherapy resistant nature of TNBC, targeted gene therapy based on studies identifying genes related to the disease is one of the frontlines for treatment of TNBC.

Till date, chemotherapy is the main stream method applied to treat breast cancer patients, even though other therapies are considered for the treatment of the patients such as targeted hormonal receptor therapy, but the effectiveness of these methods are not as much as they were hoped for treating the growing cancer[5]. In search of a more efficient way for treatment of breast cancer, TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand) has been studied extensively in the last two decades. Unlike other members of the TNF superfamily, the TNF-related apoptosis-inducing ligand (TRAIL, also known as Apo2L) possesses the unique capacity to induce apoptosis selectively in cancer cells, both in vitro and in vivo. Therefore, in recent times TRAIL is regarded as a potential anticancer agent[6,7]. TRAIL is not only present in malignant cells, but it is even expressed in some of the normal tissues such as thyroid, cerebellum, in smooth muscles of lungs and in spleen[8]. In diseased conditions TRAIL can activate both the arms of apoptosis (intrinsic and extrinsic), therefore indicating a major role in elimination of tumor cells in tumor immune surveillance[6,7]. Upregulation of DRs sensitizes the tumor cells towards TRAIL induced apoptosis. On the other hand, upregulation of surface expression of DCRs prevents the apoptotic effects of TRAIL [9].

TRAIL mediated modulation of our immune system has been one of the key features of TRAIL signalling. One of the main component of innate and adaptive immune system is the ‘Complement system’, which can activated by classical pathway (antigen-antibody complex formation system), lectin pathway and/or alternative pathway[10,11,12]. Initiation of the classical pathway is regulated by

C1q, C1r, C1s, C2, C4, CFH; lectin pathway starts with MBL, MASP2, C2, C4; and the alternative pathway involves C3, CFB, CFP, CFH, CFI, CD59. All the three pathways convert inactive C5 into the active C5 convertase, which finally generates the MAC (membrane attack complex) resulting in cell lysis[11]. Several studies have shown the complement system to be activated in response to tumor antigens[11,12]. In our study we do find an interesting link between complement system and TRAIL, which hasnot been reported before.

Furthermore, it has been proven in pre-clinical trials that TRAIL is a secure and effective way of treating breast cancer patients. However, the short-life of TRAIL and certain tumors showing resistance towards TRAIL were the major roadblock against the expected outcome of TRAIL [13]. Understanding the heterogeneity among tumor cells is very critical for clinical developments, so that the therapeutic approaches can be based on the identified resistant subpopulation and the differential regulation of cellular phenotypes[14]. Another substantial approach for treatment of breast cancer is via combination therapy, which will provide an effective alternative for present chemotherapy and also strengthen the effect of TRAIL. TRAIL in combination with Theophylline (1,3-dimethylxanthine), which is a type of methylxanthine drug that has been used for treating respiratory diseases[15] causes reduced cell viability and apoptosis. Therefore, our aim was to understand the biology of TRAIL signalling for efficient TRAIL based therapies.

3. Objectives:

3.1. Study the expression pattern of pro-apoptotic, anti-apoptotic and proliferation pathway genes on treatment with recombinant human TRAIL (rhTRAIL)

3.2. Study the molecular mechanism of apoptosis resistance on prolonged TRAIL treatment.

3.3. Study the effect of TRAIL on theophylline mediated cell death and/or mitotic arrest.

4. Methodology:

4.1. rhTRAIL protein induction and purification

TRAIL extracellular domain (aa 114 – 281) tagged with His-tag was expressed using pQE-nTR plasmid. It was obtained from Wafik El-Deiry through Addgene (plasmid #21812)[14]. The resulting 167 amino acid active TRAIL was termed as rhTRAIL (recombination human TRAIL). pET-15b control plasmid was a gift from Dr. Sugopa Sengupta, Presidency University, Kolkata. Induction and purification of rhTRAIL was performed as described by Kim et al.[14,16] . Isolated proteins were dialyzed against phosphate buffered saline with 10 mM β-mercaptoethanol. Coomassie blue gel staining and immunoblotting were used to confirm the purity of the rhTRAIL protein.

4.2. Cell Culture

Human breast adenocarcinoma, MDA-MB-231 cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained at 37ºC with 5% CO_2. Prof. Gourisankar Sa, Bose Institute, Kolkata, India has kindly gifted the MDA-MB-231 cell line.

4.3. Microarray of TRAIL treated MDA-MB-231

Total RNA from control, 50ng TRAIL treaded cells and resistant cells were isolated following the manufacturer's instructions provided with RNeasy Mini Kit (Qiagen), followed by quantification using NanoDrop spectrophotometer (Thermo Fisher Scientific). One color human gene expression experiment was performed by using Agilent Human gene expression array slide version 3, design ID (072363) for human differential expression analysis for 6 samples (each condition was analyzed in duplicate). RNA quality was analysed using Agilent 2100 Bioanalyzer (Agilent Technologies) and an input of 200ng RNA with RNI (RNA Integrity Number) value of more than 9 was used for the experiment. Experiment was preformed following the Agilent low input gene expression protocol (version 6.9). Agilent Feature Extraction software was used to obtain raw data from the scanned data Images. Data normalization, grouping, QC and statistical analysis was performed using the GeneSpring software (Agilent Technologies). Differentially expressed genes between control and 50ng TRAIL treaded cells and TRAIL resistant cells were obtained via One-way ANOVA. Benjamini-Hochberg corrected p-value cut-off was less than 0.05. Gene array analysis

was performed with the help of employees at the LCGC Life Sciences LLP, Agilent Technologies (Diagnostics and Genomics group).

4.4. DAVID analysis of identified DEGs

Database for Annotation, Visualization, and Integrated Discovery (DAVID) database is one of the most popular online tools for functional enrichment and pathway enrichment analysis of different genes in a given set of data. The GO (Gene ontology) term and KEGG pathway analysis of the selected DEGs of TRAIL treated samples was performed via DAVID.

4.5. PPI network, functional Module and Hub gene analysis

For construction of PPI network STRING (version 11.0) online database was used. The active interaction sources were set to include 'Co-occurrence', 'Co-expression', 'Experiments', 'Databases' and 'Text mining' with a confidence level of 0.4. PPI network obtained from STRING was used as an input for Cytoscape (version 3.7.2) with the cutoff of combined score > 0.9. Different modules were identified through Cytoscape plugin MCODE (default parameters were used) and ClueGO and CluePedia were also used to draw respective GO terms and KEGG pathway present in the module for visualization purpose. Hub genes were identified using the plugin CytoHubba.

4.6. Survival and correlation analysis

To understand the prognostic value of the candidate genes, Kaplan–Meier plotter (kmplot.com/analysis), an online database was used[17,18]. The software was set to provide analysis based on recurrence free survival (RFS) with respect to median of candidate gene expression in triple negative breast cancer (TNBC) patient cohort. This was performed by restricting the analysis to ER, PR and Her2 negative category of breast cancer patients. Using the above parameters, the analysis was run on 255 TNBC. Pearson's pairwise correlation analysis between the candidate hub gene and TRAIL was performed via Kaplan–Meier plotter on the same group of patient samples.

4.7. Cytotoxic (Trypan Blue) Assay

In trypan blue assay, cells were seeded in 24-well culture plate. Different rhTRAIL treatment was given for 24hrs. 10µl of 1:1 mixture of the cell suspension and trypan blue was prepared for counting through hemocytometer. Dead cells were counted under 20X objective.

4.8. Cell Survival (MTT) Assay

In MTT assay, cells were seeded in 96-well culture. Different rhTRAIL treatment was given for 24hrs. After 24hrs media was removed and incubated with SRL MTT. Then absorbance was measured at 590 nm.

4.9. Acridine Orange (AO) and Ethidium Bromide (EtBr) Staining

For AO/EtBr staining, cells were seeded in 24-well culture plates. Different rhTRAIL treatment was given for 6hrs. Then cells were stained with 8µl of AO/EtBr mix containing 100µg/ml acridine orange and 100 µg/ml ethidium bromide in PBS. Cells were viewed under fluorescent microscope.

4.10. Real-Time PCR

Resistant, sensitive and 50ng rhTRAIL treaded cells were harvested and RNA was isolated using TRIzol reagent (Ambion, Life technologies). RNA quality was determined by the A260/A280 absorbance ratio and by performing electrophoresis. 1µg of total RNA was treated with DNase followed by conversion to cDNA using reverse transcriptase as per the manufacturer's instructions (Biorad, USA). The Real-Time PCR reactions were performed for different primers listed in Table1 using SsoFast Evagreen supermix (Biorad) on PCR system 9700 (Biorad). β-actin was used as endogenous control. Threshold cycle (CT) number was measured using CFX Manager (Version 3.1.1517.0823) software (Biorad). Relative gene expression was calculated using the comparative CT method.

4.11. qPCR for Copy Number Variation

Firstly, genomic DNA was isolated of both resistant and sensitive cells. To determine the efficiency of each primers used for CNV, we have done quantitative PCR using graded dilution of genomic DNA. The dilutions are 100ng/µl, 10 ng/µl, 1 ng/µl, 0.1 ng/µl. After that using the Ct value directly, we have calculated R2 and slope of the standard curve. From the slope we have determined the primer efficiency using this formula Efficiency (%) = (10^ (-1/The Slope Value)-

1) *100. For further qPCR reactions 100ng/reaction concentration of DNA was used for each of the genes listed in Table 1. Syber green qPCR was performed with the genomic DNA and relative CDH1 and DACH1 gene copy number was calculated using the comparative CT method.

4.12. Transfection

We performed transient transfection using pcDNA3.1-E-cadherin-GFP which as a gift from Jennifer Stow (Addgene plasmid #28009). Vector control pcDNA3-EGFP was a kind gift from Dr. Abhik Saha, Presidency University, Kolkata. For transient transfection cells were seeded in 6 well plates at a density of 0.8x105 cells/well. At 24hrs, when the cells have reached a confluency of 70% old media was removed and a mixture of 4µg of DNA and 10µl of Lipofectamine™ 2000 (Invitrogen) in serum free media was added to each of the wells containing serum free media. The cells were incubated for 6hrs at 37°C, with saturated humidity and an atmosphere of 5% CO2. Then the media was removed and fresh media with serum was added. After 48hrs of transfection, cells were used for downstream experiments.

4.13. Genomic DNA isolation

The cells were seeded in T75 flask at a density of 1.2x106 cells/flask and grown for 24hrs. After trypsinization, cells were washed with PBS and taken out in fresh centrifuge tube for lysis and extraction. Genomic DNA lysis buffer (0.4M NaCl, 0.01M Tris and 0.002M EDTA) was added and vortexed vigorously. Thereafter, 10% SDS was added and gently vortexed until its mixed properly. Samples were incubated at 55^0c for 15mins, then saturated phenol was added and mixed gently. After that mixture of chloroform and isoamyl alcohol (24:1) was added and centrifuged at 6000 rpm for 5 minutes. Supernatant was collected, then again the mixture of chloroform: isoamyl alcohol was added and mixed gently. Samples were centrifuge at 6000 rpm for 5 minutes and supernatant was collected. Genomic DNA present in the supernatant was precipitated using 95% chilled ethanol. Pellets were washed thrice with 70% chilled ethanol to remove all the traces of phenol. Pellets were vacuum dried and re-suspender in sterile water.

4.14. The Cancer Genome Atlas (TCGA) analysis

For our study, we have obtained the transcript, copy number variation (CNV) and survival data for the 953 TCGA (The Cancer Genome Atlas) breast cancer samples from cBioPortal

(http://www.cbioportal.org). Similarly, transcript data for 19 breast cancer cell lines from Cancer Cell Line Encyclopedia (Novartis/Broad, Nature 2012) was downloaded through cBioPortal v3.4.4[19,20]. For transcript expression, mRNA z-score for each gene e.g. CDH1, TRAIL, DR4, DR5, DCR1 and DCR2 was obtained. Z-score indicates the number of standard deviations from the mean expression for the respective gene in the reference population. For tumor samples, the reference population is the sub-set of tumor samples out of the 953 breast cancer samples that are diploid for the respective gene[21]. The clinical data for human samples were acquired from TCGA database (https://www.cancer.gov/tcga).

4.15. ROS DCFDA assay

The cells were pre-stained with cell permeant reagent 2',7' –dichlorofluorescin diacetate (DCFDA) as per the manufacturer's instruction (Abcam, USA). Next, theophylline and control NaOH were added separately to the wells and incubated for 4-hrs. Subsequently, the cells were stained with DCFDA as per the manufacturer's instruction. The wells were read end point in a fluorescent plate reader with excitation at 485 nm and emission at 535 nm (SynergyH1, Biotek, USA).

4.16. Lipid peroxidation assay

After 6-hrs of theophylline treatment, cells were harvested followed by resuspension in 0.9% NaCl. The cells were sonicated and 100 µg of protein was taken for further analysis. Thiobarbituric acid (TBA) - Trichloroacetic acid (TCA) - HCl (0.375% TBA, 2.5% TCA dissolved in 0.1 M HCl) reagent was mixed with the protein sample and incubated at 80^{o}C for 20 min followed by centrifugation. Supernatant was collected and OD was measured at 530 nm using a spectrophotometer (Shimadzu, Japan).

4.17. NAC treatment

NAC (N-acetyl-L-cysteine) is an inhibitor of ROS. Cells were pre-treated for 1 hr with NAC (5mM) (Cat. No. 106425, Millipore, Germany) followed by treatment with theophylline for 4-hrs. This was followed by subjecting the cells to DCFDA assay, lipid peroxidation assay, trypan blue assay, cell viability assay, AO/EtBr assay and immunoblotting.

4.18. R7050 Treatment

R7050 is a cell permeable small molecule inhibitor of TNFR1 signalling. R7050 (Cat. No. 303997-35-5, Tocris, USA) was dissolved in DMSO at a final concentration of 200µM and stored in -80°C. MDA-MB-231 cells were pre-treated with R7050 for 1 hr followed by 6-hrs of theophylline treatment.

4.19. Cathepsin assay

After 6-hrs theophylline treatment, the cells were harvested and protein was isolated using RIPA (Sigma-Aldrich, now part of Merck, Germany) buffer. 20 µM Z-LR-AMC fluorogenic peptide substrate (R&D systems, USA) was added to 5 µg protein in assay buffer and incubated for 30 min. Measurement was carried out using a fluorescence plate reader with excitation at 380 nm and emission at 460 nm (SynergyH1, Biotek, USA).

4.20. Scratch Assay

Scratch assay is performed in vitro to measure cell migration. It involves 'scratching' the cell monolayer and following the movement of cells by microscope to close the scratch. The TS and TR cells were seeded in 6 well plates at a density of 1x105 cells/well in complete media. After 24hrs, when the cells have reached 90-100% confluency, the media was removed and the wells were scratched with a 200µl pipette tips in absence of any media. The wound was generated and then wells were washed with PBS. After addition of fresh serum free media (to inhibit the proliferation of cells), few photographs of the scratch area were taken for the 0hr reading, then the plate was incubated at 37°C, with saturated humidity and an atmosphere of 5% CO2. At 24hrs of incubation again scratch wound was photographed with Lieca fluorescent microscope for measurement of the gap area.

4.21. TMRE-Mitochondrial Membrane Potential Assay

10,000 cells were seeded in 96-well culture plate followed by exposure to theophylline and control NaOH. After 6 hrs incubation, TMRE (tetramethylrhodamine, ethyl ester) was added to label the active mitochondria. Experiment was carried out as per manufacturer's instruction (Cell Signalling, USA). Thereafter the samples were analysed on a plate reader at excitation of 550nm and emission of 580nm (SynergyH1, Biotek, USA). The experiment was carried out in triplicate and repeated three times.

4.22. Western Blotting

For western blot, after 6hrs of treatment cells were harvested and protein was isolated using ready to use RIPA(SIGMA) buffer. The cell extracts were separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (Millipore) and specific proteins were detected using antibodies against different genes Table 2.

4.23. Data analysis

All comparisons between groups were performed using the unpaired two-tailed t-test using SPSS statistical software (SPSS Inc., Chicago, IL, USA). P value was considered significant at and below 0.05.

5. Results:

5.1. Objective 1: Study the expression pattern of pro-apoptotic, anti-apoptotic and proliferation pathway genes on treatment with recombinant human TRAIL (rhTRAIL)

5.1.1. Identification of genes that were differentially expressed at the transcript level between the TRAIL treated and control MDA-MB-231 breast cancer cells

MDA-MB-231 breast cancer cells were treated with 50ng/ml rhTRAIL or given mock treatment (control). We observed rhTRAIL to cause increased cell death as observed by trypan blue assay (Figure 1a) and elevated levels of cleaved caspase-3 compared to control MDA-MB-231 cells (Figure 1b). To identify the genes involved in TRAIL induced apoptosis microarray was performed. During microarray analysis, a total of 32,045 probes were studied out of which 4721 probes were found to be significantly different between the TRAIL treated and control cells. These 4721 probes consisted of 3633 transcripts having an NM ID (Genbank accession number), among which we observed 3463 number of unique transcripts genes. Next, the transcripts were shortlisted based on fold change of greater than 1.5 and less than -1.5. This resulted in identification of 306 unique transcripts that were observed to be differentially expressed between the rhTRAIL treated and control breast cancer cells. These 306 transcripts consisted of 300 unique differentially expressed genes (DEGs). Out of the 300 DEGs, 202 DEGs were observed to be upregulated and

98 DEGs were found to be downregulated in the rhTRAIL treated cells in comparison to the control cells (Figure 1c).

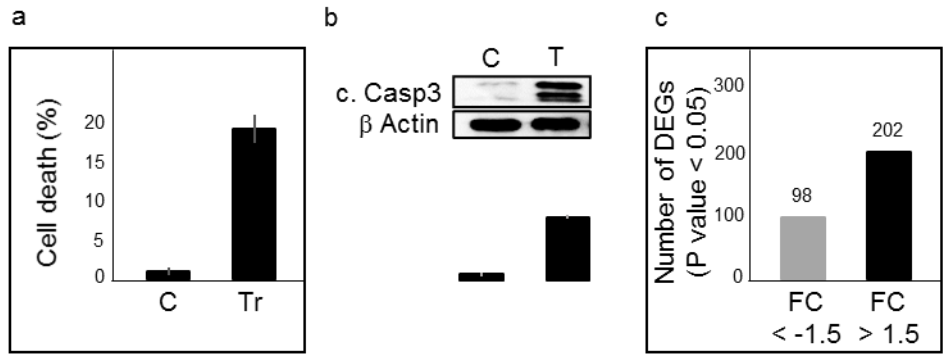


Figure 1. *rhTRAIL induced cytotoxicity and identification DEGs from microarray data. (a) Cell death was measured using trypan blue assay in MDA-MB-231 with 50ng/ml rhTRAIL treatment. (b) Cleaved caspase-3 protein was measured using western blotting. β-actin was used as an endogenous control. (c) Number of upregulated and downregulated DEGs with a fold change of >1.5, < -1.5. (C: control; Tr: rhTRAIL; * indicates p≤0.05).*

5.1.2. Gene Ontology and KEGG pathway analysis of the 300 differentially expressed transcripts through DAVID gene functional classification tool

For preliminary understanding of the biological importance of the 300 candidate transcripts, GO analysis was performed using DAVID online algorithm. We observed 10 most significant GO (Figure 2a). We observed that the genes belonging to 'regulation of complement activation' biological process are also a part of the significant CC and MF as shown in Figure 2b. Furthermore, KEGG pathway enrichment analysis showed hsa04610:Complement and coagulation cascades and hsa05150:Staphylococcus aureus infection to be significantly enriched by 7- and 9-fold, respectively, in rhTRAIL treated cells. We observed complement genes i.e. C1R, C1S, C2, C4B, CFB, CFH, CFI to be common between the significant KEGG pathways (Figure 2c).

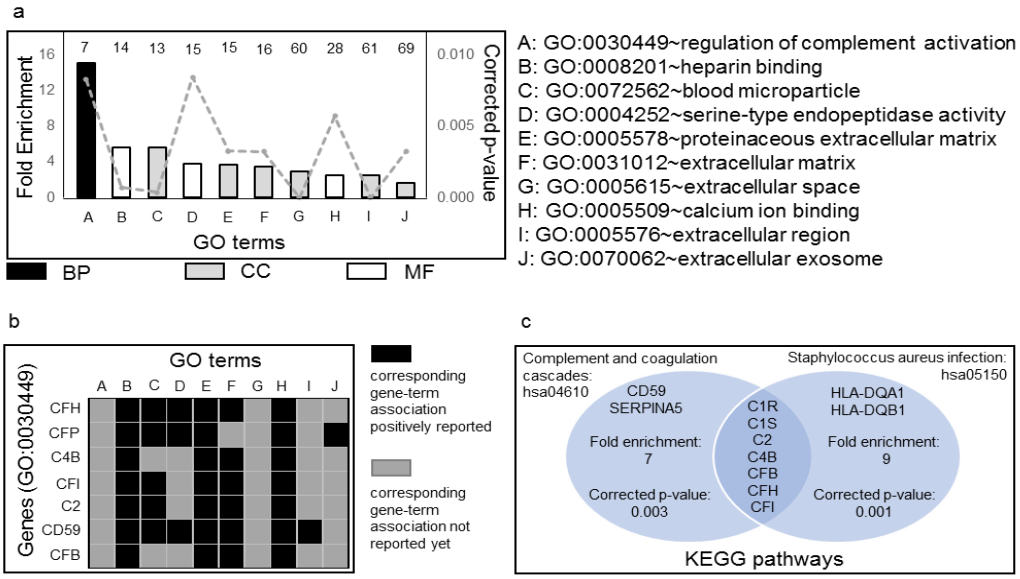


Figure 2. *GO enrichment analysis of 300 DEGs and Venn diagram of KEGG pathway analysis. (a) Bar diagram of Significant GO terms (b) Heat map of the candidate DEGs present in the most enriched GO term in the other significant GO terms. (c) Venn diagram of common DEGs between two significant KEGG pathways*

5.1.3. Module and hub gene analysis of DEGs to identify candidate genes related with TNBC survival prediction

STRING database was used to generate PPI (Protein Protein Interaction) network using the 300 DEGs. This was followed by analysis of the PPI network by Cytoscape software that resulted in identification of 208 nodes and 419 edges (Figure 3a). Next, the MCODE plugin of Cytoscape identified modules from the STRING PPI network. The top module (module 1) consisted of 8 nodes, namely, C59, C2, C4B, CFB, C1R, C1S, CFP and ADAMTS13 (Figure 3b). Next, hub gene analysis was performed via Cytoscape plugin CytoHubba in order to identify functionally central genes among the 297 300 DEGs. We identified the top 8 hub genes with highest maximal clique centrality (MCC) consist of the following complement genes - CFH, CFB, CFP, C4B, C1R, C1S, CD59 and C2. We observed CFB, C2 and C4B to be the hub genes present in module 1, GO term

biological processes GO:0030449 as well as in genes that are common to KEGG pathways hsa04610 and hsa05150 (Figure 3c). Therefore, CFB, C2 and C4B hub genes were considered as candidate genes for further study. In order to identify whether the candidate genes are prognostically relevant in predicting disease outcome in TNBC patients, online Kaplan–Meier plotter was used. We observed CFB (Affymetrix probe ID: 211920_at) (HR = 0.59(0.38-0.9), logrank p = 0.015) and C2 (Affymetrix probe ID: 203052_at) (HR = 0.6(0.39-0.92), logrank p = 0.019) to significantly predict recurrence free survival (RFS) in TNBC patients (n=255) (Figure 3d, 3e). Unfortunately, Kaplan–Meier plotter did not have an Affymetrix probe that was exclusive for C4B. Therefore, the C4B gene was not considered for further study. We observed that high expression of CFB and C2 complement genes increased the probability of relapse free survival in TNBC patients ($p < 0.05$).

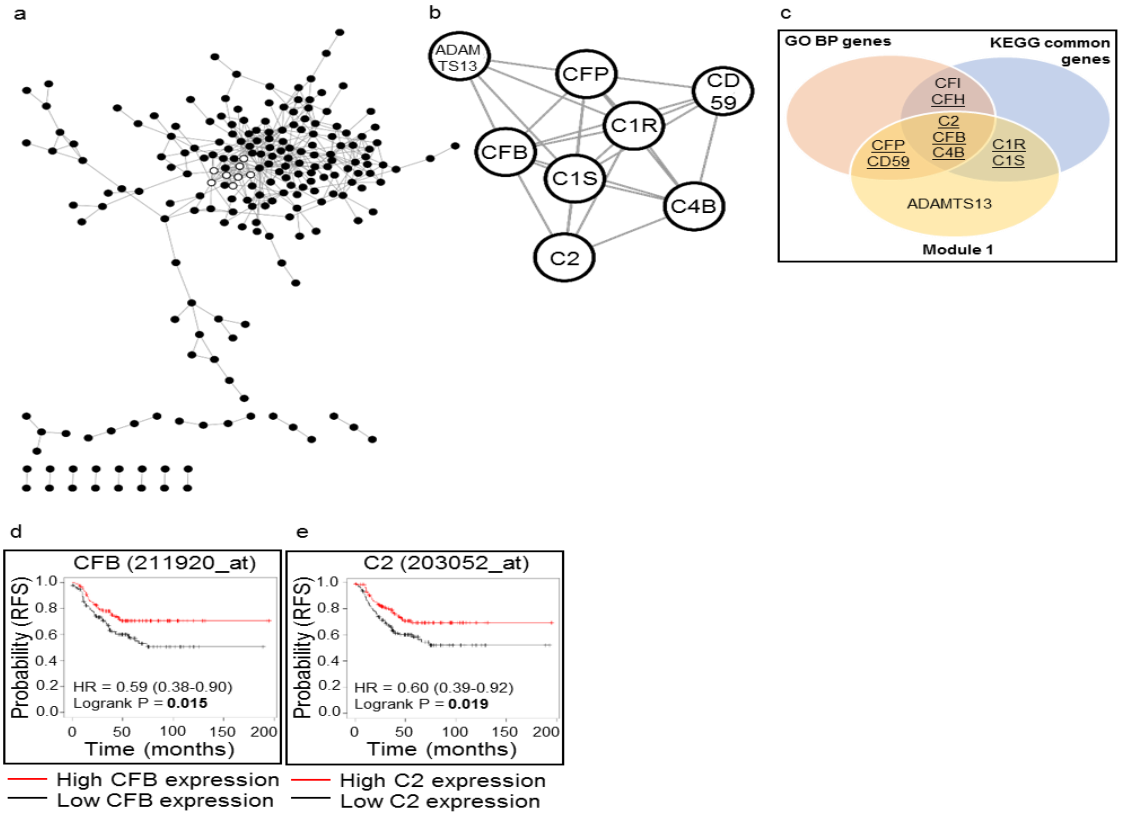


Figure 3. *Module analysis and identification of candidate genes. (a) PPI network of identified DEGs. (b) Module with highest score of 6.85 and consist of 8 edges. (c) Venn diagram of common*

genes between most enriched GO term, KEGG pathway and top module. (d, e) Survival analysis of CFB and C2 in TNBC patients was obtained from KM plotter (HR = 0.59(0.38-0.90), log-rank p- 0.015; HR = 0.60(0.39-0.92), log-rank p- 0.019).

5.1.4. Analysis of CFB hub genes in the context of TRAIL in triple negative breast cancer patients

To further understand the role of CFB and C2 in triple negative breast cancer in relation to TRAIL, we conducted correlation analysis through online Kaplan–Meier plotter. We observed CFB (Pearson's correlation coefficient, $r = 0.13$, $p < 0.05$) but not C2 (Pearson's correlation coefficient, $r = 0.09$, $p > 0.05$) to be positively correlated with TRAIL in these 255 TNBC patient samples (Figure 4a). Furthermore, since CFB was observed to be correlated with TRAIL we performed the Kaplan–Meier analysis on these 255 TNBC patients after segregating them with respect to median TRAIL expression. We observed high CFB to significantly predict increased probability of RFS in high TRAIL group (HR = 0.37 (0.17-0.79), logrank p = 0.008) but not in low TRAIL group (HR = 0.8 (0.47-1.36), logrank p = 0.410) (Figure 4b).

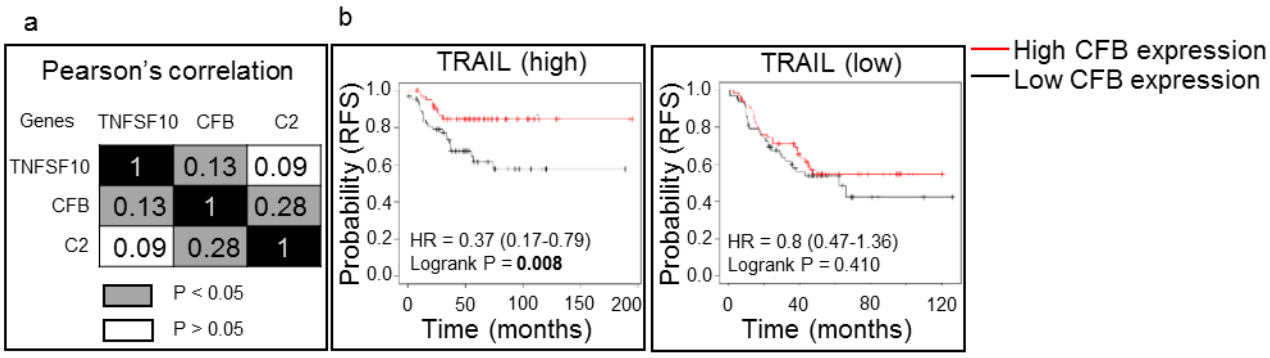


Figure 4. *Validation of CFB and C2 relation with TRAIL treatment. (a) Positive correlation between CFB and TRAIL expression in the TNBC (r = 0.13, p <0.05) and positive correlation between C2 and TRAIL expression in the TNBC (r =0.09, p < 0.05). (b) Survival analysis of CFB in TNBC patients divided as per high (HR = 0.37(0.17-0.79), log-rank p- 0.008) and low (HR = 0.8(0.47-1.36), log-rank p- 0.410) expression of TRAIL obtained from KM plotter.*

5.1.5. Conclusion

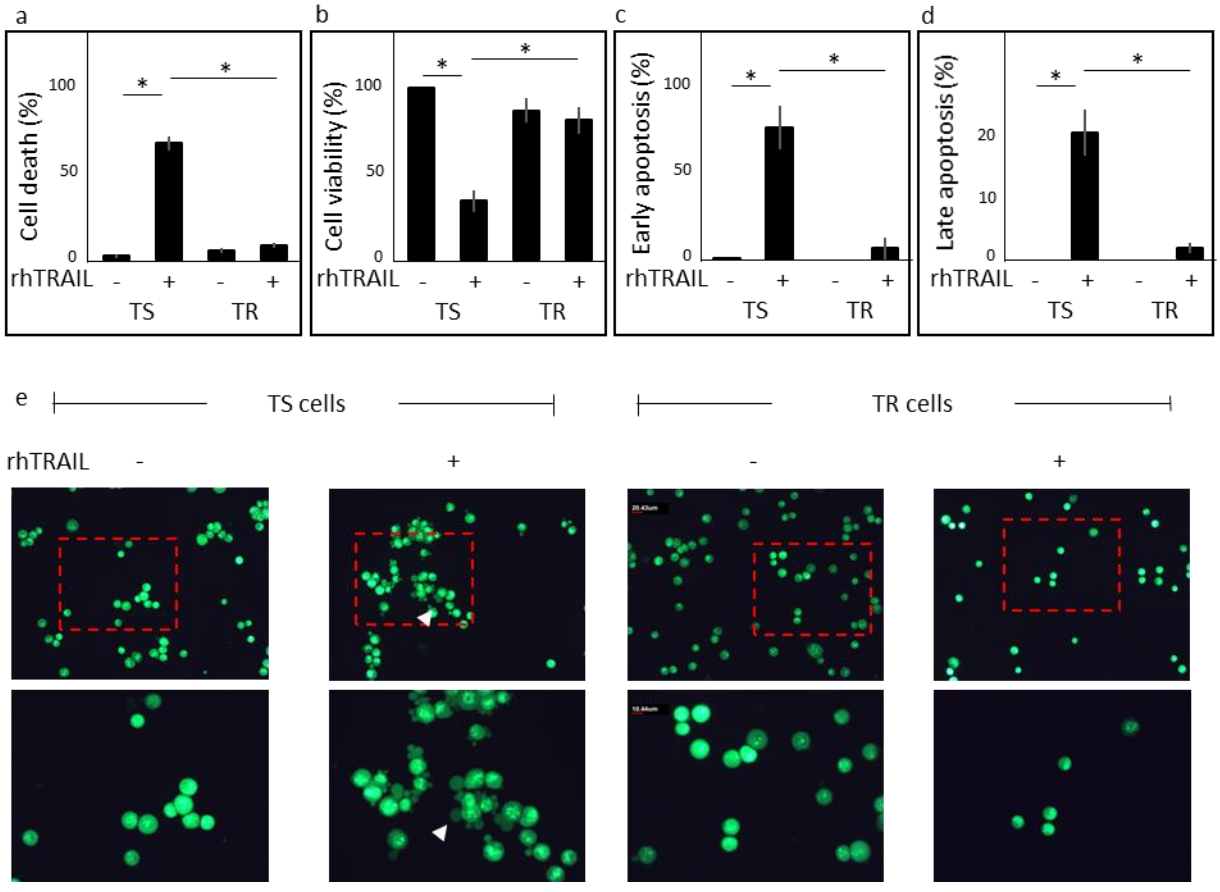
- Triple Negative Breast Cancer (TNBC) cell line MDA-MB-231 treated with 50ng/ml rhTRAIL influences GO terms related with Complement pathway and extracellular region. KEGG pathways includes Staphylococcus aureus infection and Complement and coagulation cascades.
- CFB and C2 are common between GO terms, KEGG pathway and the most significant module, which shows positive prognostic values for TNBC. Both of these hub genes are positively corelated with TRAIL in 255 TNBC patients
- When TNBC patient group was divided as per TRAIL expression, the group with high TRAIL expression shows high CFB was related with better relapse free survival. Even CFB has shown to co-express with TRAIL
- We identified complement factor B (CFB) to have a favorable prognostic value in triple negative breast cancer patient expressing high levels of endogenous TRAIL. Thus, our study indicates the complement system to be involved in promoting TRAIL mediated cell death which needs to be explored further in the context of triple negative breast cancer.

5.2. Objective 2: Study the molecular mechanism of apoptosis resistance on prolonged TRAIL treatment

5.2.1. Selection of TRAIL resistant (TR) cells from a population of TRAIL sensitive (TS) MDA-MB-231 cells

Breast cancer cell line, MDA-MB-231, has been observed to be sensitive to TRAIL mediated cytotoxicity[5,22,23,24]. In the present study, we have established a TRAIL resistant breast cancer cell line, TR (TRAIL resistant), by exposing the TS (TRAIL sensitive) MDA-MB-231 cells with 800ng/ml rhTRAIL (IC99: 800 ng/ml) for 24hrs. The surviving rhTRAIL resistant population of cells (1% of the initial population) were allowed to grow in a culture media without rhTRAIL till the confluency reached 70% (approximately 14 days). Thereafter, the TR cells were treated with 500ng/ml rhTRAIL on every alternate passage. All the experiments were performed after the cells had been grown in media without rhTRAIL for at least one passage. To confirm rhTRAIL resistance of MDA-MB-231 cells, trypan blue dye-exclusion test, MTT assay and AO/EtBr staining was performed after 500ng/ml rhTRAIL and mock treatment. TR cells showed

significantly lower trypan blue positive dead cells compared to TS cells after 500ng/ml rhTRAIL treatment (Figure 5a). Similarly, MTT assay showed a significantly higher cell viability in TR cells compared to TS cells after rhTRAIL treatment (Figure 5b). In Acridine orange/Ethidium Bromide (AO/EtBr) staining assay, membrane blebbing, which represents early apoptosis, increased significantly after 6hrs of rhTRAIL treatment in comparison to mock treatment for TS cells. However, TR cells showed significantly reduced membrane blebbing on rhTRAIL exposure in comparison to rhTRAIL-treated TS cells (Figure 5c, 5e). Similarly, late apoptosis, as indicated by the presence of EtBr-stained red nucleus, showed a significant increase on rhTRAIL treatment in TS cells but not in TR cells when compared to mock treatment. Moreover, late apoptosis observed in TR cells was significantly lower compared to TS cells in the presence of rhTRAIL (Figure 5d, 5f)



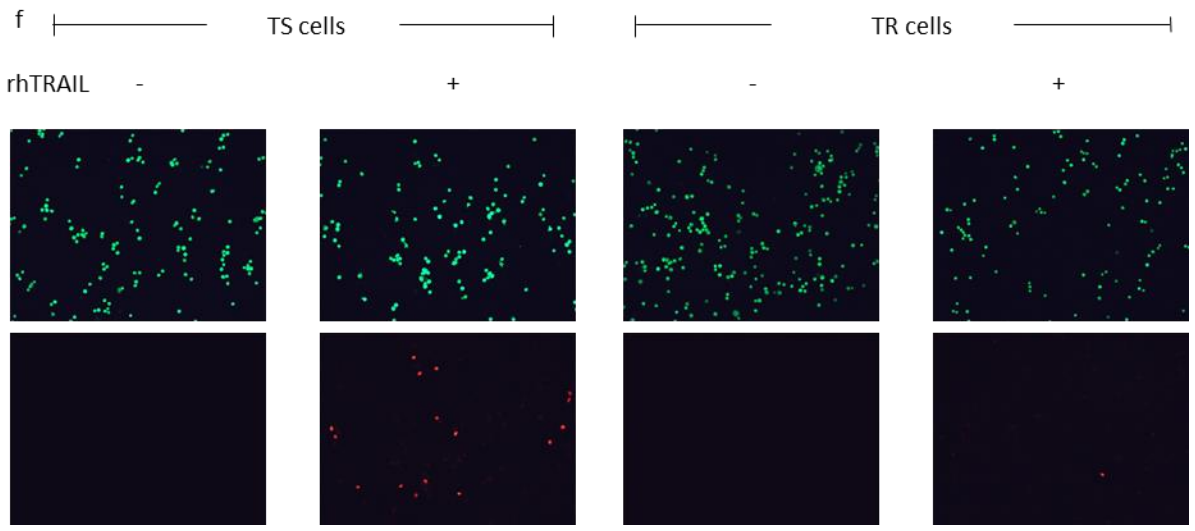


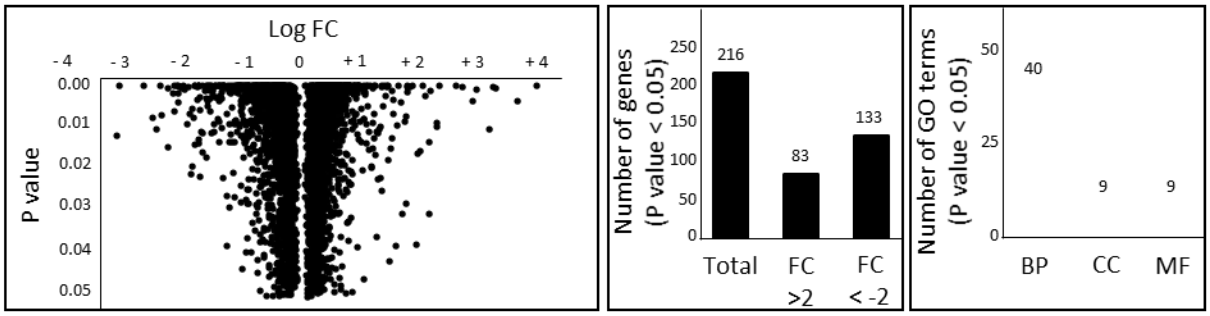
Figure 5. *MDA-MB-231 TR cell population show resistance towards TRAIL mediated cytotoxicity on exposure to rhTRAIL. Inherent TRAIL resistant (TR) cells were selected from a population of TRAIL sensitive (TS) MDA-MB-231 cells using 800 ng/ml (IC99) rhTRAIL. The TR cells and TS cells were maintained in cell culture with 500ng/ml rhTRAIL and mock treatment, respectively, on every alternate passage. Next, both TS cells and TR cells were treated with rhTRAIL (500ng/ml) for 24hrs. Cytotoxicity was measured by (a) trypan blue assay and (b) MTT assay. AO/EtBr staining was used to measure (c) early apoptosis and (d) late apoptosis after 6-hrs of rhTRAIL (500ng/ml) treatment in TS and TR cells. (e) Representative image of cells showing membrane blebbing (arrow) after staining with AO dye. (f) Representative image of cells showing EtBr stained cells after AO/EtBr staining. All experiments were repeated three times. Representative experiment has been shown in the figure.*

5.2.2. Microarray analysis of DEGs among rhTRAIL resistant and sensitive population of cells through DAVID software

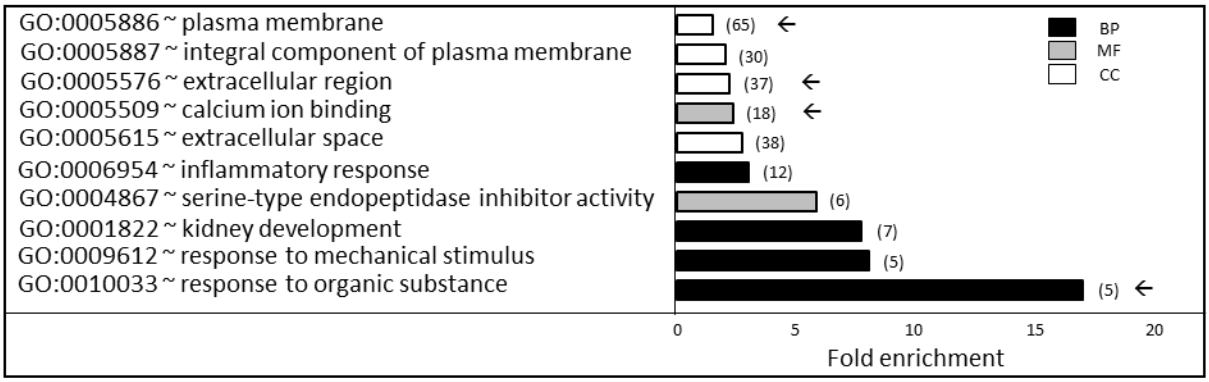
To identify the DEGs responsible for rhTRAIL resistance in MDA-MB-231 cells, microarray analysis was performed using TR cells where TS cells were used as control. Out of 32,045 probes that were analyzed, 6,598 probes were found to be significantly different between the TR and TS cells. 6,598 probes consisted of 5,247 genes having NM ID out of which we observed 4907 number of unique DEGs. The distribution of DEGs is shown in Figure 6a. Next, the genes were shortlisted based on fold change of greater than 2 and less than -2 to obtain a set of 216 DEGs. Out of the 216

DEG genes, we observed 83 genes to be upregulated and 133 genes to be downregulated in TR cells in comparison to control TS cells (Figure 6b).

These 216 DEGs were analyzed using DAVID. GO enrichment analyses via DAVID indicated that there are 40 significant enriched GO terms belonging to biological process (BP), 9 enriched GO terms belonging to cellular components (CC) and 9 enriched GO terms belonging to molecular function (MF) in TR cells (Figure 6c). The top 10 significant GO terms along with fold-enrichment has been shown in figure 6d. Analysis of the 216 DEGs through KEGG pathway of DAVID identified 7 significant pathways (Figure 6e).



d



e

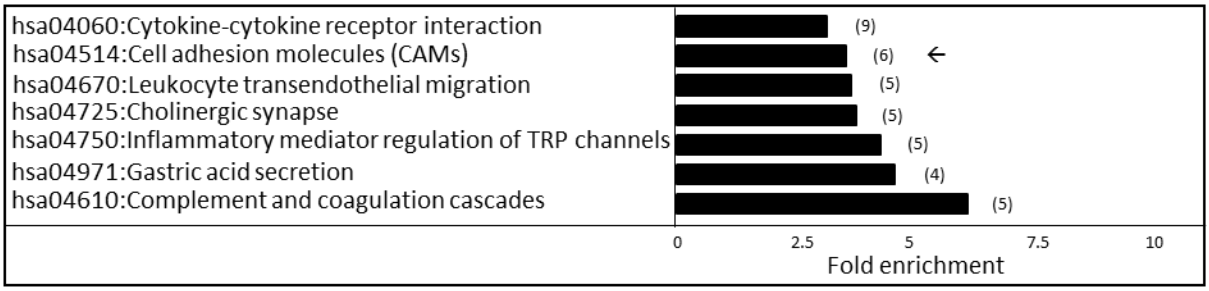


Figure 6. *Analysis of microarray data for identification and functional characterization of candidate differentially expressed genes (DEGs) in MDA-MB-231 TR cells. Microarray experiment was carried out in duplicate for TS and TR cells. (a) The volcano plot shows 4907 significant DEGs that are present in TR cells in comparison to TS cells. (b) The distribution of significant DEGs (216 genes) in TR cells that show a fold change (FC) of greater than 2 and less than -2. (c) The number of significant GO terms obtained through DAVID by analyzing the DEGs shown in figure 2(b) belonging to biological process (BP), cellular component (CC) and molecular function (MF). (d) The top 10 significant GO terms and (e) significant KEGG pathways obtained by analyzing the DEGs shown in figure 2(b). The number in parenthesis beside each bar indicates the gene count corresponding to the respective GO and KEGG pathway category. Arrow indicates that CDH1 belongs to that particular category.*

5.2.3. Analysis of PPI network for identification of Hub DEGs

Previously identified 216 DEGs were used for PPI analysis through STRING database where 216 nodes and 231 edges were identified. Next, after analyzing the PPI network using Cytoscape we identified 139 hub genes (Figure 7a). These hub genes (nodes) were arranged based on degree centrality and betweenness centrality. We identified CDH1 to be the hub gene with the highest degree centrality with connections to 18 nodes (Figure 7b). CDH1 was also found to have highest betweenness centrality. This indicated that CDH1 might also play an important role in the interconnections of other node pairs in the context of TRAIL resistance in breast cancer.

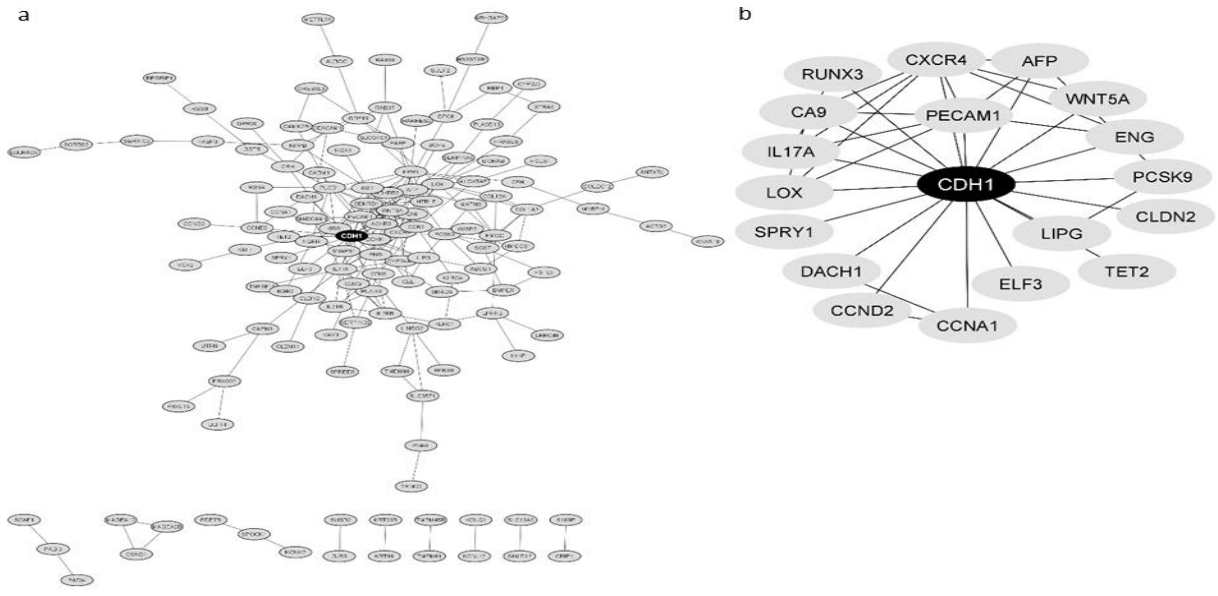


Figure 7. *PPI network analysis. (a) PPI network was constructed with 139 hub genes. (b) PPI network of CDH1 hub gene with 18-degree centrality, showing interaction of CDH1 with 18 different node genes. In both (a) and (b), CDH1 has been shown in black circle and the other hub genes have been shown in grey circle.*

5.2.4. Decreased expression of CDH1 transcript might be due copy number variation at the CDH1 locus in rhTRAIL resistant cells

CDH1 is a well-known tumor suppresser gene that plays an important role in apoptosis, cell proliferation and migration[25,26,27,28,29]. Validation of microarray data by quantitative real-time PCR showed CDH1 transcript to be downregulated by 0.43-fold in TR cells with respect to TS cells ($P \leq 0.05$) (Figure 8a). To investigate the reason behind the downregulation of CDH1 mRNA, copy number variation (CNV) analysis was carried out for the CDH1 locus in TR cells and TS cells. We identified copy number deletion at the CDH1 locus in TR cells in comparison with TS cells (Figure 8b). Analysis of the 953 TCGA breast cancer (BRCA) patients revealed 28.12% patients to be diploid at the CDH1 locus. The remaining 62.12% and 28.12% patients showed deletion and amplification, respectively, at the CDH1 locus. Further investigation of the TCGA BRCA patients revealed that the mRNA expression of CDH1 gene was significantly lower in patients having copy number deletion compared to patients having diploid copy of the CDH1 gene (Figure 8c). This indicated that copy number deletion might be one of the factors responsible for decreased expression of the CDH1 transcript in the TR cells.

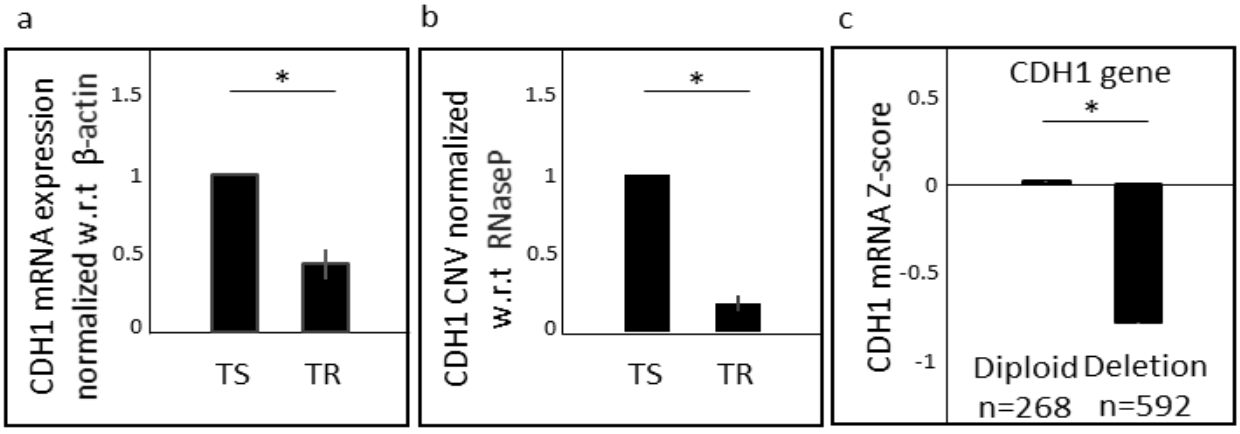


Figure 8. *MDA-MB-231 TR cells show reduced CDH1 expression. (a) Transcript analysis was carried out for TS and TR cells using real-time PCR. β-actin was used as an endogenous control. (b) Genomic DNA was analyzed to study copy number variation (CNV) at CDH1 locus in TS and TR cells. RNaseP was used as an endogenous control. Graphs shows average expression of three independent experiments normalized with respect to (w.r.t) the respective endogenous controls. (c) CNV analysis of CDH1 mRNA z-score in 953 TCGA patient samples.*

5.2.5. Overexpression of CDH1 increases apoptosis in rhTRAIL resistant cells compared to rhTRAIL sensitive cells

Furthermore, western blot analysis showed CDH1 protein to have lower abundance in TR cells in comparison to TS cells (Figure 9a). Therefore, in order to determine the role of CDH1 in regards to rhTRAIL resistance in MDA-MB-231 breast cancer cells, CDH1 (or control plasmid) was over expressed in TR and TS cells (Figure 9b, 9c). We observed that overexpression of CDH1 in TR cells made them prone to cell death and lowered their cell viability in presence of rhTRAIL compared rhTRAIL-treated TR cells transfected with empty vector ($P \leq 0.05$) (Figure 9d, 9e). Furthermore, early and late apoptosis was significantly increased in CDH1 overexpressing, rhTRAIL-treated TR cells in comparison to TR cells that were transfected with control vector and treated with rhTRAIL (Figure 9f, 19g). Additionally, cleaved caspase-3 was observed to increase in rhTRAIL-treated TR cells that were overexpressing CDH1 in comparison to rhTRAIL-treated control TR cells that did not overexpress CDH1 (Figure 9h). Taken together, we can conclude that overexpression of CDH1 in TR cells sensitized them to rhTRAIL induced apoptosis.

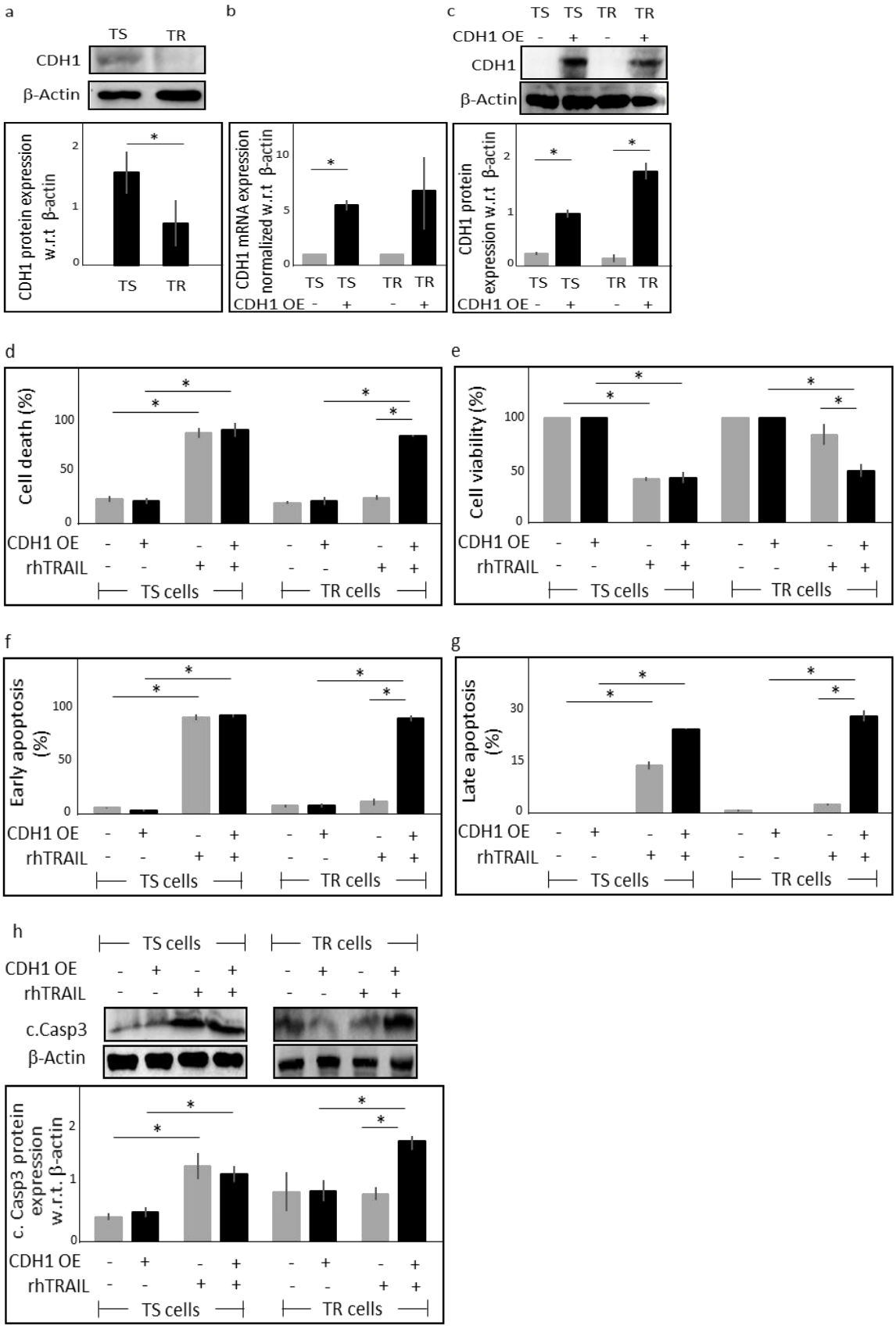

a
TS
TR
CDH1
β-Actin
CDH1 protein expression w.r.t β-actin
b
CDH1 mRNA expression normalized w.r.t β-actin
TS TS TR TR
CDH1 OE - + - +
c
TS TS TR TR
CDH1 OE - + - +
CDH1
β-Actin
CDH1 protein expression w.r.t β-actin
d
Cell death (%)
CDH1 OE
rhTRAIL
TS cells
TR cells
e
Cell viability (%)
f
Early apoptosis (%)
g
Late apoptosis (%)
h
c.Casp3
β-Actin
c. Casp3 protein expression w.r.t. β-actin

Figure 9. *Overexpression of CDH1 sensitizes TR cells towards TRAIL mediated toxicity. 48hrs posttransfection of CDH1 (or control plasmid) in TS and TR cells (a) CDH1 transcript was measured using realtime PCR and (b) CDH1 protein was measured using western blotting. β-actin was used as an endogenous control. Both quantitative PCR (qPCR) and immunoblot graphs shows average expression of two independent experiments normalized with respect to (w.r.t) endogenous control. (c) Trypan blue assay was carried out to measure cell death and (d) MTT assay was performed to measure cell viability in CDH1 (or control plasmid) overexpressing TS and TR cells after 24hrs of 500ng/ml rhTRAIL treatment. All experiments were carried out in triplicate and repeated three times. Representative experiment has been shown in the figure. (e) Early apoptosis and (f) late apoptosis was measured by AO/EtBr assay in CDH1 (or control plasmid) overexpressing TS and TR cells after 6hrs of 500ng/ml rhTRAIL treatment. All experiments were carried out in triplicate and repeated three times. Representative experiment has been shown in the figure. (g) Western hybridization showing cleaved caspase-3 in control plasmid and CDH1 transfected TS and TR cells after 6hrs of rhTRAIL (500ng/ml) treatment. β-actin was used as an endogenous control. Graph shows average expression of three independent experiments normalized with respect to (w.r.t) endogenous control.*

5.2.6. TRAIL resistant breast cancer tumor samples showed reduced CDH1 expression in comparison to TRAIL sensitive tumor samples

Next, TRAIL resistant and TRAIL sensitive breast cancer cell lines were identified from published literature [30,31,32,2]. Thereafter, mRNA z-score of TRAIL and its four receptors were used to calculate a numerical value, (TRAIL + DR4 + DR5)/ (TRAIL + DCR1 + DCR2), which we termed as the apoptosis/survival ratio (A/S ratio)[33,34,35]. We observed that the TRAIL resistant breast cancer cell lines have significantly low A/S ratio in comparison to TRAIL sensitive cell lines which show high A/S ratio (Figure 10a). Thus, we could conclude from the cell line study that the A/S ratio is capable of predicting the sensitivity of breast tumor cells towards TRAIL treatment. Subsequently, 953 breast cancer patient samples obtained from TCGA database were subdivided into TRAIL sensitive (high A/S ratio) and TRAIL resistant (low A/S ratio) groups. Interestingly, we observed that the TRAIL resistant group showed significantly reduced CDH1 mRNA expression in comparison to the sensitive group (Figure 10b). We further observed CDH1 transcript expression to be significantly lower in the TRAIL resistant group in Stage I, II and III

breast tumors in comparison to the TRAIL sensitive group (Figure 10c). This indicates that low CDH1 expression is consistent throughout breast cancer in TRAIL resistant group irrespective of tumor stage.

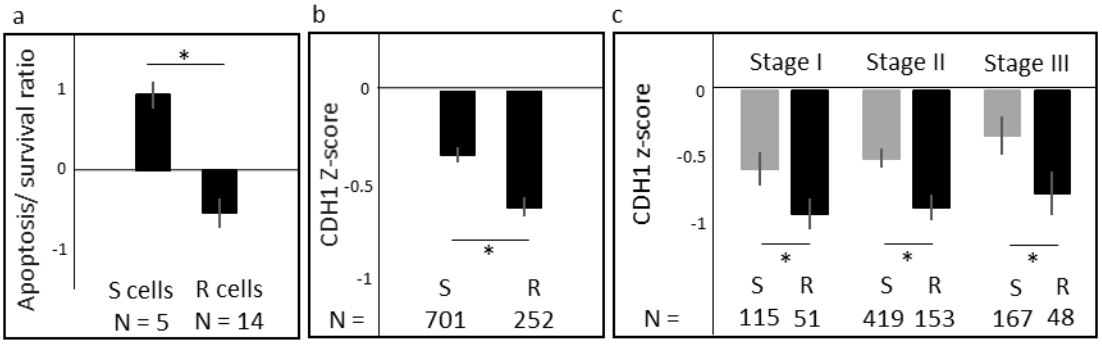


Figure 10. *TCGA breast cancer patient group with low A/S ratio shows reduced expression of CDH1 mRNA level. (a) Apoptosis/ survival (A/S) ratio using mRNA z-score of (TRAIL+DR4+DR5)/ (TRAIL+DCR1+DCR2) of TRAIL-sensitive and -resistant cell lines. (b) CDH1 mRNA expression of TRAIL-sensitive and -resistant patient groups. (c) CDH1 z-score of breast cancer tumor stage I, II, and III of the TRAIL-sensitive and -resistant patient groups.*

5.2.7. Conclusion

- In a tumor cell population there are always certain cells which are inherently resistant to TRAIL induced apoptosis. After treatment with high(800ng) dose of rhTRAIL, the resistant cell population was isolated and use for microarray experiment.
- We observed 216 differentially expressed genes belong to 43 GOTERM BP, 9 GOTERM CC and 7 KEGG PATHWAY. The most significant Go term ‘response to organic substance’ showed a 16.6-fold enrichment and comprised of WNT5A, CRIP1, S100P, CDH1, ABCG1.
- After PPI network and Hub gene analysis, CDH1 was found to be the gene with the highest degree centrality and maximum betweenness centrality. CDH1 was downregulated in TRAIL resistant (TR) cells.
- CNV analysis using genomic DNA (gDNA) showed copy number deletion at the CDH1 gene locus of TR cells compared to TS cells. Analysis of the 953 TCGA breast cancer samples showed CDH1 copy number loss.

- Overexpression of CDH1 in TR cells increased cell death and reduced cell viability in response to rhTRAIL treatment in comparison to control TR cells. rhTRAIL treatment also increases the expression of cleaved caspase-3 in the resistant cells in comparison to the control non-treated resistant cells.
- Breast cancer patients show low CDH1 transcript expression in TRAIL resistant patient group in comparison with the TRAIL sensitive group.

5.3. Objective 3: Study the effect of TRAIL on theophylline mediated cell death and/or mitotic arrest.

5.3.1. Exposure to theophylline causes reduced viability in breast cancer cells and tumorigenic human embryonic kidney cells but not normal fibroblast cells

We examined the ability of theophylline to influence cell viability in MDA-MB-231 breast cancer cells. MTT assay showed theophylline to significantly reduce cell viability in breast cancer cells as compared to control (Figure 11a). Similarly, theophylline significantly reduced cell viability in HEK293, tumorigenic human embryonic kidney cells (Figure 11b). Furthermore, 'normal' immortalized mouse embryonic fibroblast cells, NIH3T3, did not show any significant difference in cell viability on theophylline treatment (Figure 11c).

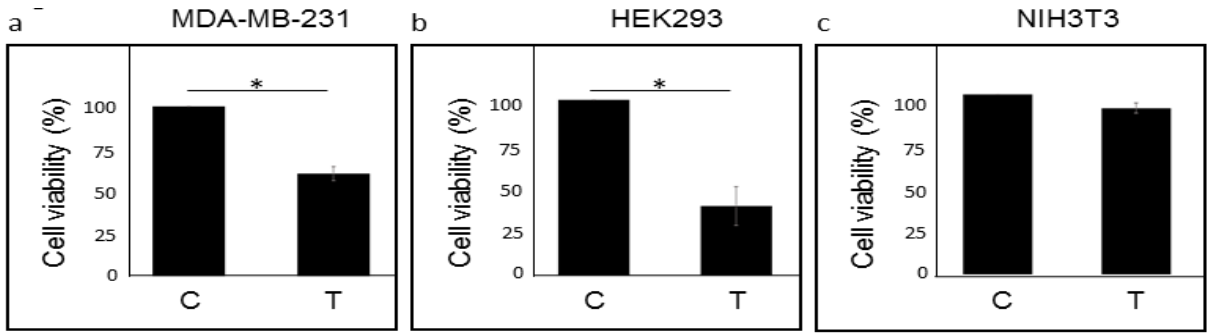


Figure 11. *Theophylline reduces cell viability in tumorigenic cells but not normal cells. Cell viability was measured using MTT assay in (a) MDA-MB-231, (b) HEK293, and (c) NIH3T3 cells after treatment with theophylline for 24-hrs. All experiments were carried out in triplicate and repeated three times. Representative experiment has been shown in the figure. (C: control; T: theophylline; * indicates p≤0.05).*

5.3.2. Theophylline induces cell death, DNA damage and apoptosis in MDA-MB-231 cells

We examined the ability of theophylline to induce cytotoxicity in MDA-MB-231 breast cancer cells. Theophylline showed a significant increase in cell death as compared to control (Figure 12a) using trypan blue assay. Next, alkaline comet assay was performed to identify whether DNA damage was also involved in theophylline mediated cell death. We observed that MDA-MB-231 cells on treatment with theophylline for 6 hrs showed significantly higher total DNA damage compared to control ($P<0.05$) (Figure 12b). Similarly, neutral comet assay showed significantly higher amounts of double strand as well as single strand DNA breaks in theophylline treated cells in comparison to control (Figure 12c). Staining of theophylline treated cells with acridine orange and ethidium bromide showed some typical morphological changes that are associated with apoptosis. Membrane blebbing, which represents early apoptosis, increased significantly after 6 hrs of theophylline treatment as compared to control ($P<0.05$) (Figure 12d, 12e). Similarly, late apoptosis, as indicated by the presence of EtBr-stained red nucleus, showed an increase on theophylline treatment ($P<0.05$) (Figure 12f).

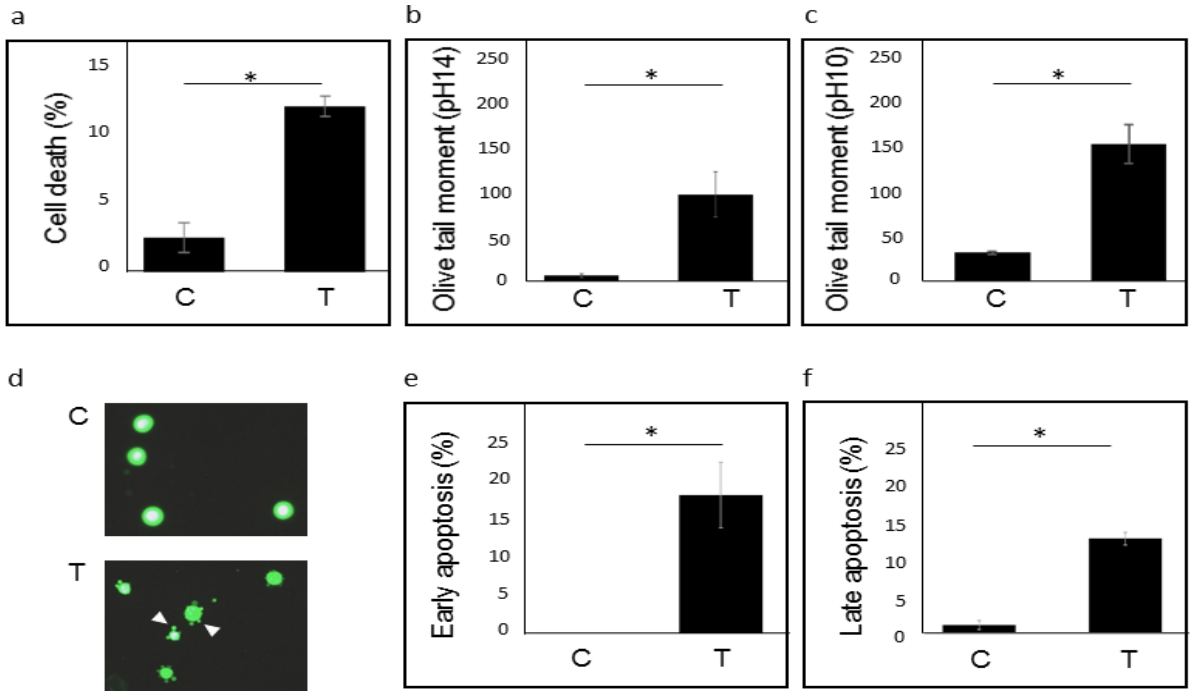


Figure 12. *Theophylline causes cytotoxicity in MDA-MB-231 breast cancer cells. (a) Cytotoxicity was measured by trypan blue assay after 24-hrs of theophylline treatment. (b) Total DNA damage and (c) dsDNA damage are represented as Olive tail moment after performing alkaline and neutral*

*comet assay after 6-hrs of theophylline treatment, respectively. (d) Representative image of cells showing membrane blebbing (arrow) after staining with AO dye. AO/EtBr staining was used to measure (e) early apoptosis and (f) late apoptosis after 6-hrs of theophylline treatment. All experiments were carried out in triplicate and repeated three times. Representative experiment has been shown in the figure. (C: control; T: theophylline; * indicates p≤0.05).*

5.3.3. Theophylline initiates TNFR1 mediated activation of caspases in MDA-MB-231 cells

In order to explore the molecular mechanism activated by theophylline we studied TNFR1 death receptor mediated apoptotic pathway. We found TNFR1 transcript and protein to be significantly upregulated on theophylline treatment in MDA-MB-231 breast cancer cells and tumorigenic HEK293 cells (Figure 13a, 13b,13c, 13d). We further observed that exposure of MDA-MB-231 cells to theophylline caused an increase in the levels of cleaved caspase-8, -9 and -3 protein (Figure 13e,13f,13g). Furthermore, we observed reduction in mitochondrial membrane potential in theophylline treated cells (Figure 13h). R7050, a small molecule inhibitor is a known antagonist of TNFR1. It functions by blocking the binding of TNFR1 to TRADD and RIP1 thereby blocking TNFR1 mediated apoptosis[36]. To establish the fact that TNFR1 is one of the important receptors to mediate theophylline induced cell death, R7050 was given in combination with theophylline to the MDA-MB-231 cells. We observed significant increase in cell viability after treatment with R7050 and theophylline compared to cells that had been treated theophylline alone (Figure 14i). Similarly, the cleaved caspase-8, -9 and -3 levels also decreased in R7050 treated cells that were exposed to theophylline as compared to theophylline alone (Figure 13j,13k,13l).

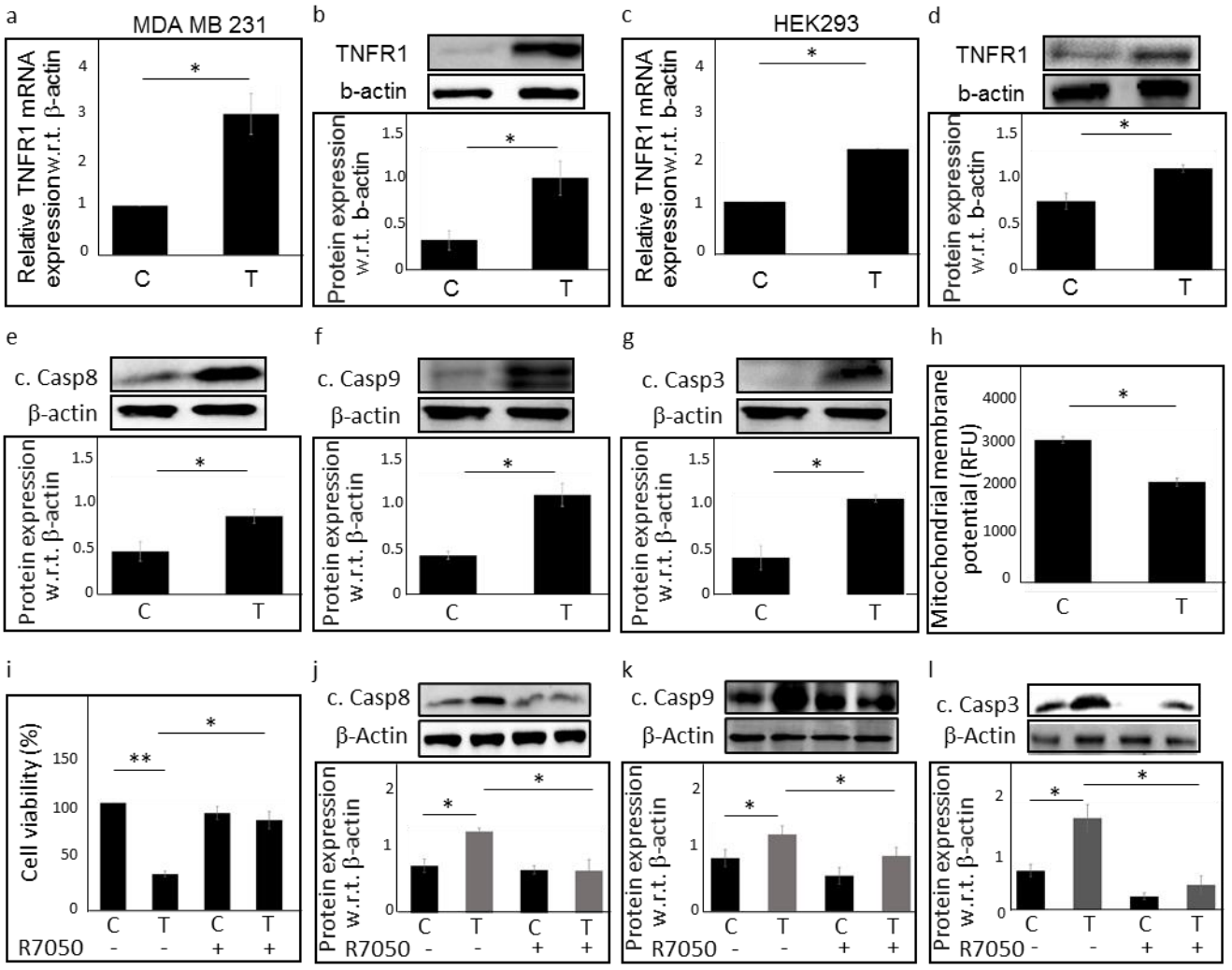


Figure 13. *Theophylline activates TNFR1 mediated apoptosis. After 6-hrs of theophylline treatment, qPCR as well as immunoblotting was performed for TNFR1 in MDA-MB-231 (a, b) and HEK293 (c, d) cells. Immunoblotting of cleaved caspase-8 (e), cleaved caspase-9 (f) and cleaved caspase-3 (g) apoptotic proteins was carried out after 6-hrs theophylline treatment. β-actin was used as an endogenous control. Graphs corresponding to immunoblots show average protein expression of three independent experiments normalized with respect to (w.r.t) endogenous control. (h) Mitochondrial membrane potential was measured using TMRE dye after 6-hrs theophylline treatment (i) Cytotoxicity was measured by MTT assay with and without R7050 after 24-hrs of theophylline treatment. (j) Level of cleaved caspase-8, -9 and -3 proteins in control and theophylline treated cells were measured by immunoblotting in presence and absence of R7050. β-actin was used as an endogenous control. (C: control, T: theophylline; * indicates p≤0.05).*

5.3.4. ROS contributes to the cytotoxic effects of theophylline in MDA-MB-231 breast cancer cells

Breast cancer cells when treated with theophylline showed higher amount of ROS generation (Figure 14a). Since ROS is capable of initiating peroxidation of cellular lipids, we tried to investigate whether theophylline treatment also showed elevated lipid peroxidation. Indeed, theophylline was found to significantly increase lipid peroxidation with respect to control (Figure 14b). Furthermore, to investigate if the theophylline induced toxicity is through ROS mediated pathway, we treated the cells with a ROS inhibitor, NAC. On treatment with NAC we observed reduced generation of ROS as well as decreased lipid peroxidation in theophylline treated cells compared to cells that were not treated with NAC ($p \leq 0.05$) (Figure 14c,14d). Next, we observed that NAC-mediated ROS reduction significantly reduced the number of dead cells and increased cell viability even in the presence of theophylline (Figure 14e,14f). Similarly, the percentage of cells undergoing apoptosis reduced on administration of NAC along with theophylline in comparison to theophylline alone ($p \leq 0.05$) (Figure 14g,14h). Additionally, we observed NAC treated cells to show reduced levels of TNFR1 and cleaved caspase-3 protein in theophylline exposed cells compared to cells that were treated with only theophylline ($p \leq 0.05$) (Figure 14i,14j).

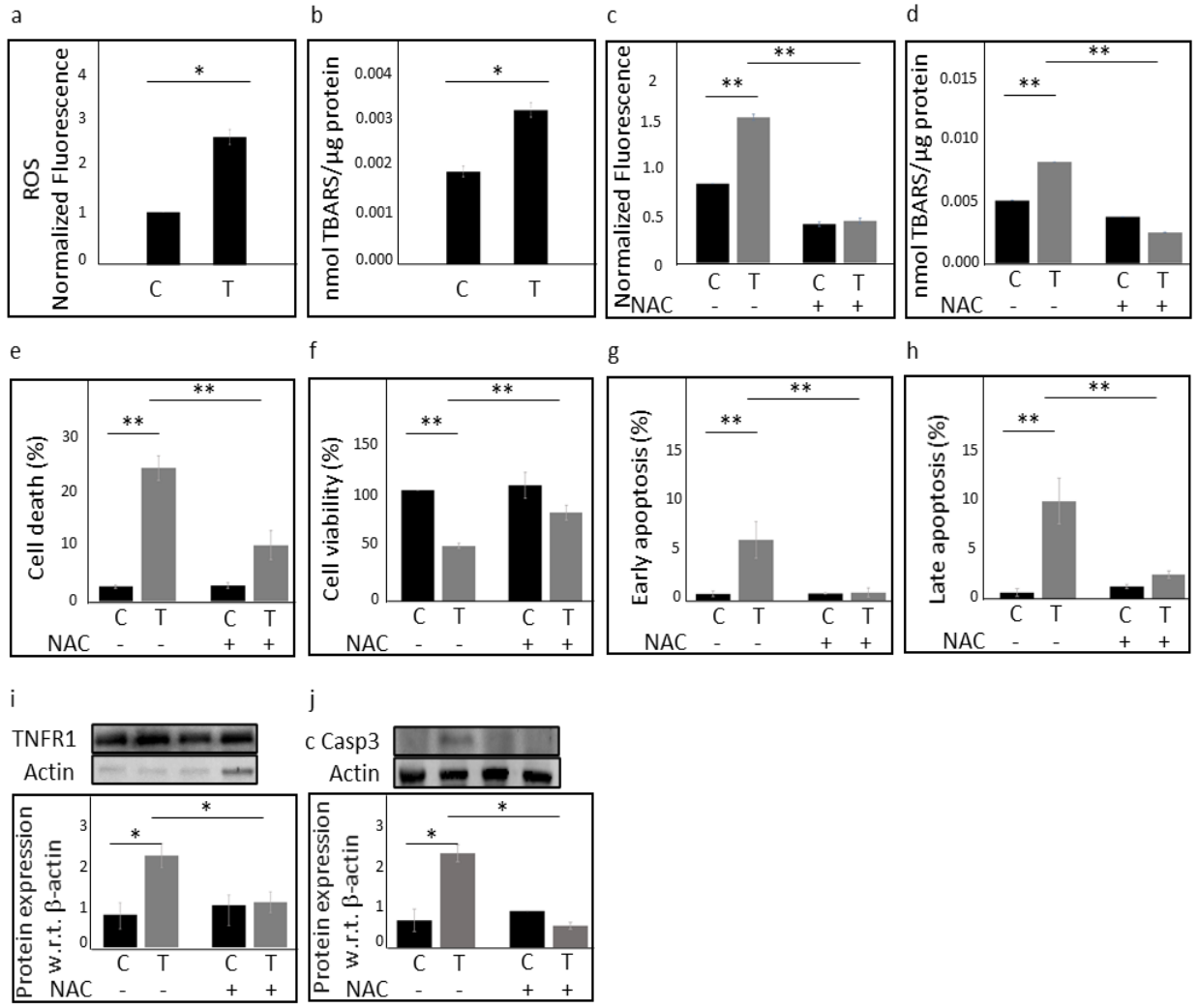


Figure 14. *Theophylline induces ROS production inhibition of which results in decreased TNFR1 mediated apoptosis in MDA-MB-231 breast cancer cells (a) ROS was measured in presence of DCFDA reagent after 4-hrs theophylline treatment. (b) Oxidative damage to lipids was measured by TBARS assay after 6-hrs of theophylline treatment. (c) The intracellular ROS production was measured in control and theophylline treated cells by DCFDA assay in presence and absence of ROS inhibitor, NAC. (d) TBARS assay was used to measure the levels of lipid peroxidation in cells treated with/without theophylline in presence and absence of NAC. Percentage of cell death (e) and cell viability (f) in cells treated with/without theophylline in presence and absence of NAC was assessed using trypan blue and MTT assay, respectively. Early (g) and late (h) apoptosis was assayed using AO/ EtBr dye in control and theophylline treated cells in presence and absence of NAC. Levels of TNFR1 (i) and cleaved Caspase-3 (j) proteins in control and theophylline treated cells were measured by immunoblotting in presence and absence of NAC. The graphs show the*

*average protein expression from three independent experiments normalized with respect to (w.r.t) endogenous control. (C: control; T: theophylline; * indicates p≤0.05).*

5.3.5. Theophylline causes an increase in cathepsin activity via TNFR1 in MDA-MB-231 breast cancer cells

Cathepsin, a lysosomal cysteine protease, is known to be involved in TNFα mediated apoptosis[37]. Interestingly, we found the activity of cathepsin proteases to increase in MDA-MB 231 cells after 6-hrs of theophylline treatment (p≤0.05) (Figure 15a). When the TNFR1 was blocked via R7050 we saw a significant decrease in active cathepsin level after theophylline treatment (Figure 15b).

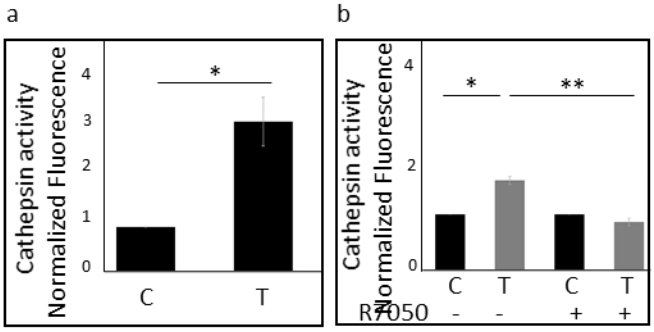


Figure 15. *Theophylline treatment causes increased cathepsin activity which is reduced on inhibition of TNFR1 in MDA-MB-231 cells. (a) The activity of cathepsin was measured as the amount of fluorescent AMC dye after 6-hrs of theophylline treatment (b) The cathepsin activity showed reduction on treatment of the cells with TNFR1 inhibitor, R7050. All experiments were carried out in triplicate and repeated three times. Representative experiment is shown in the figure (C: control; Tr:rhTRAIL; T: theophylline; Tr+T: rhTRAIL+Theophylline; * indicates p≤0.05).*

5.3.6. rhTRAIL in combination with theophylline causes cytotoxicity in breast cancer cell line

As we have already established that theophylline has cytotoxic effect on MDA-MB-231 TNBC cell line, now we are trying to study the effect of rhTRAIL in combination with theophylline. Here we observed via trypan blue and MTT assay that combination of 150ng/ml of rhTRAIL with

10mM of theophylline induces significant cell death in comparison to rhTRAIL and theophylline alone after 24hrs of treatment($p \leq 0.05$) (Figure 16a,16b). Late apoptosis caused by combination treatment was observed by EtBr-stained red nucleus after 6hrs of treatment, experiment showed similar results as the earlier assay, in combination the percentage of late apoptotic cells were greater that those of rhTRAIL and theophylline treated($p \leq 0.05$) (Figure 16c).

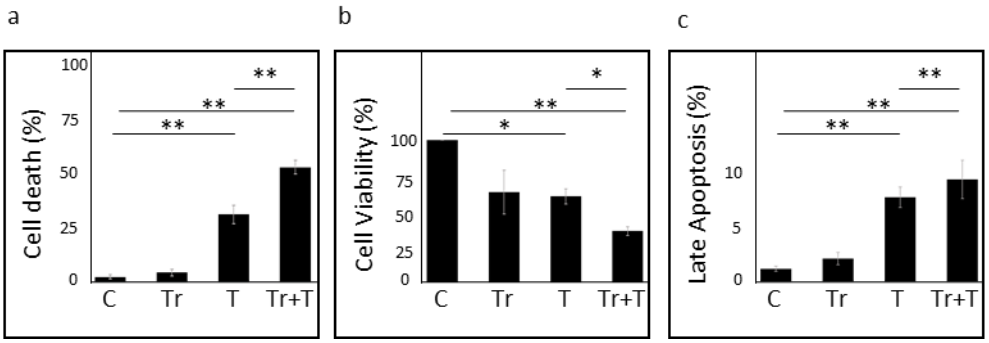


Figure 16. *Combination treatment decreases cell viability and migration in MDA-MB-231 cells. (a) Cytotoxicity was measured by trypan blue assay after 24-hrs of rhTRAIL, theophylline and combination treatment.(b) MTT assay was done after 24hrs of treatments. AO/EtBr staining was used to measure (c) late apoptosis after 6-hrs of respective treatments. (C: control; Tr:rhTRAIL; T: theophylline; Tr+T: rhTRAIL+Theophylline; * indicates p≤0.05).*

5.3.7. Combination treatment of theophylline and rhTRAIL induced both extrinsic and intrinsic pathways of apoptosis better than theophylline or rhTRAIL alone

We further observed that theophylline in combination with rhTRAIL significantly increased cleaved caspase-8 protein level after 6hrs of treatment in comparison to theophylline or rhTRAIL alone (Figure 17a). As TRAIL is known for inducing both the branches of apoptosis, we studied the intrinsic apoptotic genes such as cleaved caspase-9 and the final effector protein of apoptosis pathway, caspase-3. Both of the cleaved caspases are significantly upregulated in cells treated with theophylline and rhTRAIL alone as well as in combination compared to control cells. Also, the cleaved caspase-9 and caspase-3 in combination is higher than those of theophylline and rhTRAIL treated sample (Figure 17b, 17c).

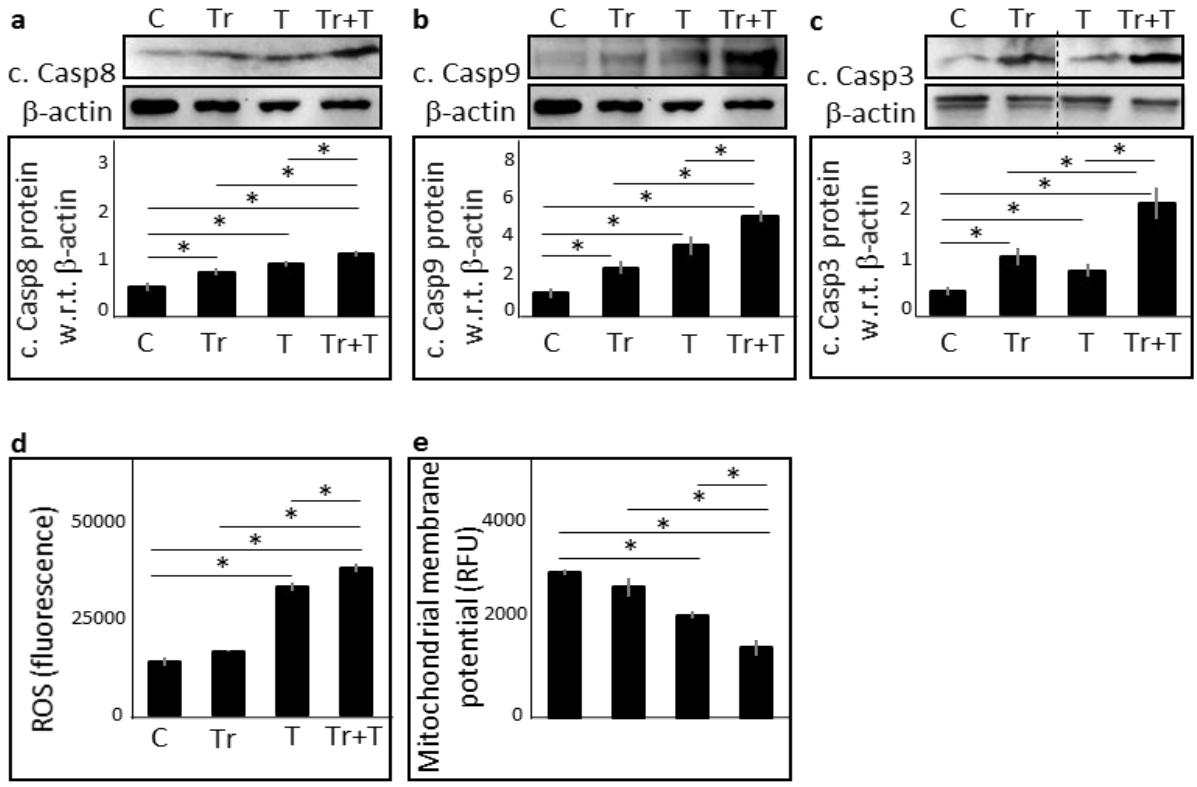


Figure 17. *Combination treatment triggers intrinsic apoptotic pathway. .(a, b,c) Level of cleaved caspase-8, -9 and -3 proteins in 6hrs treated cells were measured by immunoblot. β-actin was used as an endogenous control.(d) ROS was measured in presence of DCFDA reagent after 4-hrs theophylline treatment and combination treatment. (e) Mitochondrial membrane potential was measured using TMRE dye after 6-hrs of rhTRAIL, theophylline and combination treatment (C: control; Tr:rhTRAIL; T: theophylline; Tr+T: rhTRAIL+Theophylline; * indicates p≤0.05).*

5.3.8. Theophylline in combination with rhTRAIL induced high ROS production along with reduced mitochondrial membrane potential in comparison to theophylline or rhTRAIL alone

To gain insight into the mechanism of cytotoxicity mediated via theophylline in combination with rhTRAIL, we observed the level of ROS production in MDA-MB-231 cells treated with theophylline and/or rhTRAIL. ROS generation was found to be significantly upregulated in cells treated with combination treatment, in comparison to rhTRAIL and theophylline separately (Figure 17d). Additionally, we observed mitochondrial membrane potential to be lower in combination treatment compared to single treatment of rhTRAIL and theophylline (Figure 17e).

5.3.9. Theophylline upregulated DR5 expression in combination with rhTRAIL with respect to single treatment

To understand the mechanism by which apoptosis is induced by combination treatment, we performed western blot analysis for DR5 protein. We found that expression of DR5 was increased significantly on addition of theophylline with rhTRAIL with respect to MDA-MB-231 cells that were exposed to theophylline or rhTRAIL alone (Figure 18).

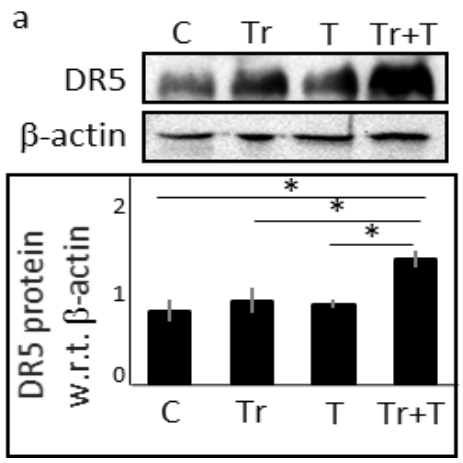


Figure 18. *Upregulation of death receptors by rtTRAIL in combination with theophylline. (a) Expression levels of DR5 protein in 6hrs treated cells were measured by immunoblot. β-actin was used as an endogenous control. (C: control; Tr:rhTRAIL; T: theophylline; Tr+T: rhTRAIL+Theophylline; * indicates p≤0.05).*

5.3.10. Conclusion

- Theophylline shows cytotoxic effect on triple negative MDA-MB-231 breast cancer cell line and on embryonic kidney cell line HEK293t but it fails to show any effect on normal cell line NIH3T3.
- Theophylline treatment shows significant amount of DNA damage and lipid peroxidation as a result of presence of high amount of ROS. Inhibition of ROS production through NAC (N-acetyl-l-cysteine) significantly decreased the cell death caused by theophylline. ROS reduction by NAC causes downregulation of TNFR1 mediated apoptosis via reduction in active caspase 3.

- Theophylline upregulates expression of death receptors like TNFR1 in both MDA-MB-231 and HEK293 cell lines, thereby upregulating the protein expressions of cleaved caspase-8, caspase-9 and caspase-3 in the breast cancer cell line. In presence of TNFR1 inhibitor R7050 the expression of cleaved caspases decreases.
- Theophylline also enhances activity of cathepsin in MDA-MB-231 cell line.
- Theophylline in combination with rhTRAIL increases cell death and reduces cell viability in comparison to theophylline or rhTRAIL alone. Combination treatment also causes decreased cell migration.
- rhTRAIL in combination with theophylline upregulates caspase-8, 9 and 3, along with ROS generation and depolarisation of mitochondrial membrane potential.
- Combination treatment upregulates protein level of Death receptor 5(DR5). Therefore, we can conclude that TRAIL in combination with theophylline could be a promising therapeutic approach for treatment of breast cancer.

6. Overall Conclusion

- Taken together, our initial results suggest that treating patients with TRAIL might help in increasing the CFB-mediated complement response against tumor.
- As tumors developing resistance against TRAIL is one of the major concerns regarding TRAIL therapy, it is required that we understand the nature of TRAIL resistant cells. Our study shows that inherent rhTRAIL resistant cells have downregulated CDH1 in comparison to the rhTRAIL sensitive cells. Overexpression of CDH1 in the rhTRAIL resistant cells makes them sensitive to rhTRAIL treatment thereby paving a way for exploring TRAIL based therapies for breast cancer treatment.
- Furthermore, to develop other ways of treating breast cancer efficiently with TRAIL, we investigated the potential of combination therapy of theophylline with rhTRAIL as it has been previously reported that theophylline induces apoptosis in breast cancer cells. First, we studied the mechanism of theophylline inducing cell death in breast cancer cells, then we observed the effect of theophylline in combination with TRAIL. Interesting we observed significantly higher cell death in combination samples, this provides us with a new opportunity of treating cancer cells effectively with TRAIL.

7. Significance of the study

Our study shows that rhTRAIL is capable of inducing complement pathway of innate and adaptive immune system in TNBC cell line, which might help in eradication of tumor cells. As TRAIL resistance is one of the main challenges faced during clinical trial, our study shows that CDH1 expression level plays an important role in regulating TRAIL resistance. This indicates that CDH1 can be also used as a biomarker for TNBC patients during TRAIL mediated therapy. We found that theophylline, which is known drug for treating asthma patients and is readily available in the market can induce cell death in cancer cell lines. This can be a promising alternative treatment for breast cancer in this era of chemotherapy. We have also shown that rhTRAIL used in combination with theophylline increases the effectiveness of TRAIL on TNBC cell line, which can be further used in treating TNBC patients.

8. Future Directions

- To understand the molecular mechanism by which CFB in responsible for apoptosis in rhTRAIL treated breast cancer cells and to understand its role in rhTRAIL resistant breast cancer cells.
- To analyse the expression levels and Risk free survival (RSF) with respect to CFB and CDH1 in triple negative breast cancer patient population from Indian subcontinent.

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Kolkata 700073

11. Table1.

Gene	Primer sequence
qPCR	
CDH1 Forward	5’- GATTTTTCGGCAGTTCAAGC-3’
CDH1 Reverse	5’- CCACCCCCAAAGAAAATACA -3’
TNFR1 Forward	5’-GAAGTTGTGCCTACCCCAGA-3’
TNFR1 Reverse	5’-GGGATAAAAGGCAAAGACCA-3’
β-Actin	5’-CCCAGCACAATGAAGATCAA-3’
β-Actin	5’-ACATCTGCTGGAAGGTGGAC-3’
qPCR for Copy Number Variation	
CDH1 Forward	5’- TTGCTACTGGAACAGGGACA-3’
CDH1 Reverse	5’-CCCCGTGTGTTAGTTCTGCT-3’
DACH1 Forward	5’- GTGGAAAACACCCCTCAGAA-3’
DACH1 Reverse	5’-CTTGTTCCACATTGCACACC-3’
RNaseP	5’- AGATTTGGACCTGCGAGCG-3’
RNaseP	5’-GAGCGGCTGTCTCCACAAGT-3’

12. Table2.

Antibody	Catalog Number
Caspase-3	CST #9665

Caspase-8	CST #D35G2
Caspase-9	CST #9502
TNFR1	CST #3736
CDH1	CST #3195
DR5	CST #8074
β-Actin	Biobharati LifeScience, India

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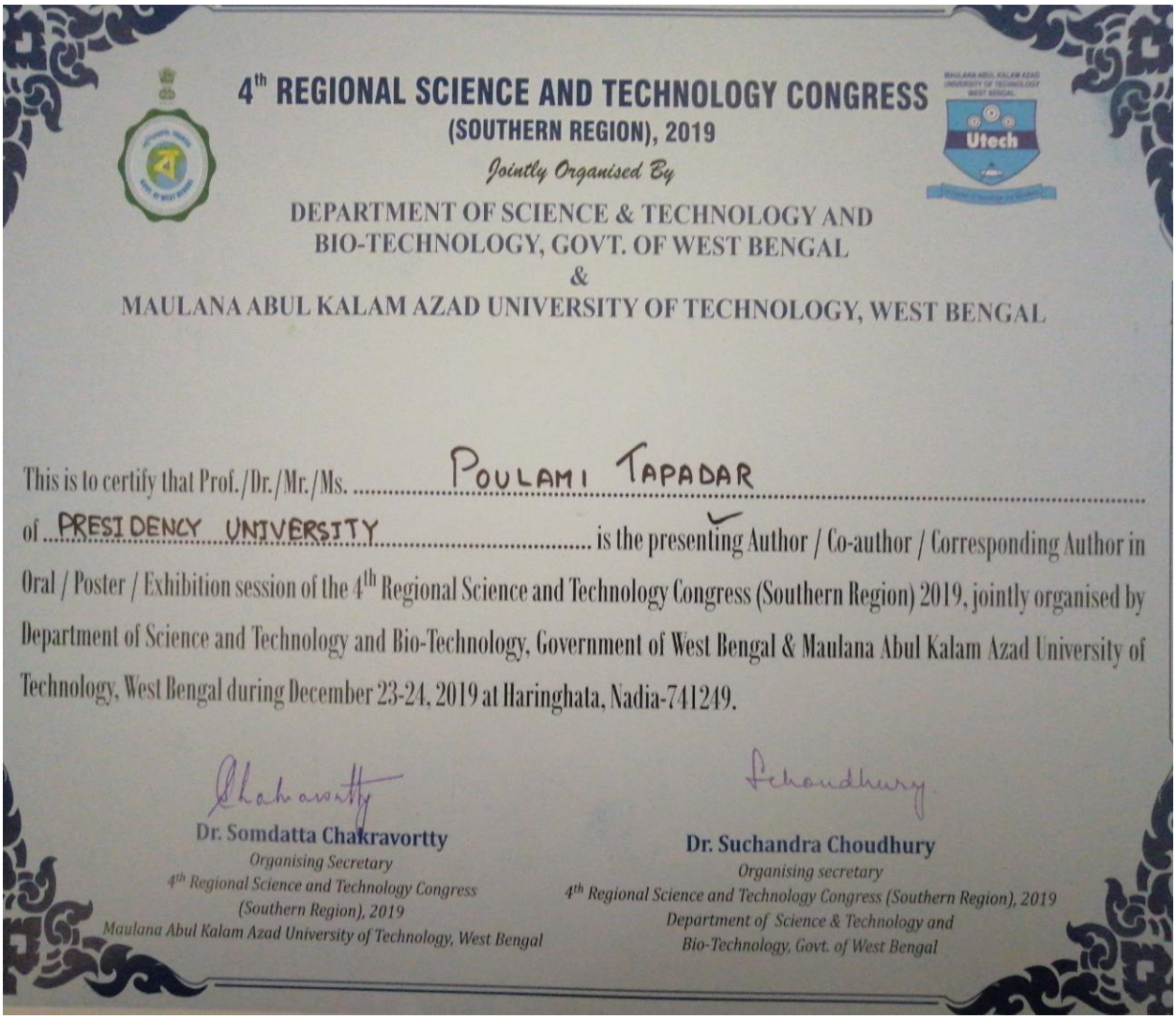

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The Editorial Team

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Enhanced Expression of DR5 is Responsible for Increased Cytotoxicity of Theophylline in Combination with rhTRAIL in MDA-MB-231 Breast Cancer Cells

Running title: Molecular mechanism of theophylline along with rhTRAIL in breast cancer

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Abstract:

Background: Theophylline has been reported to induce cytotoxicity and cell cycle arrest in cancer cells. On the other hand, TRAIL, a secretory ligand, is known for its unique ability to induce cell death only in tumor cells. In the present study we elucidated the mechanism behind the cytotoxic effect of theophylline in combination with rhTRAIL (recombinant human TRAIL) on cancer cell line MDA-MB-231.

Methods: Cytotoxicity of theophylline in combination with TRAIL was measured via trypan blue assay and MTT assay. Protein levels were assessed using western hybridization. ROS levels was measured using DCFDA and Mitochondrial Membrane Potential (MMP) assay was conducted using TMRE.

Results: We observed theophylline in combination with rhTRAIL to be significantly cytotoxic to the cancer cells in comparison to theophylline and rhTRAIL alone. Next, western hybridization showed combination treatment to upregulate cleaved form of caspase-8, 9 and 3, in comparison to the cells treated with rhTRAIL and theophylline alone. Theophylline in combination also increased the levels of ROS and reduced mitochondrial membrane potential. Interestingly, combination treatment increased the protein level of DR5 (death receptor 5), sensitizing the cells towards TRAIL induced apoptosis.

Conclusion: Theophylline in combination with TRAIL significantly increases cytotoxicity in MDA-MB-231 breast cancer cell line when compared to theophylline and rhTRAIL alone via upregulation of DR5 levels.

Keywords: Theophylline; rhTRAIL; apoptosis; ROS; DR5

Key message: Upregulation of DR5 by treatment of MDA-MB-231 breast cancer cells with theophylline along with rhTRAIL makes them more prone to apoptosis in combination therapy.

RESEARCH ARTICLE

Exploring the Molecular Mechanism of Cinnamic Acid-Mediated Cytotoxicity in Triple Negative MDA-MB-231 Breast Cancer Cells

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Abstract: ***Background*:** Cinnamic acid (CA), also known as 3-phenyl-2-propenoic acid, is a naturally occurring aromatic fatty acid found commonly in cinnamon, grapes, tea, cocoa, spinach and celery. Various studies have identified CA to have anti-proliferative action on glioblastoma, melanoma, prostate and lung carcinoma cells.

***Objective*:** Our objective was to investigate the molecular mechanism underlying the cytotoxic effect of CA in killing MDA-MB-231 triple negative breast cancer cells.

***Methods*:** We performed MTT assay and trypan blue assay to determine cell viability and cell death, respectively. Comet analysis was carried out to investigate DNA damage of individual cells. Furthermore, AO/EtBr assay and sub-G1 analysis using flow cytometry were used to study apoptosis. Protein isolation followed by immunoblotting was used to observe protein abundance in treated and untreated cancer cells.

***Results*:** Using MTT assay, we have determined CA to reduce cell viability in MDA-MB-231 breast cancer cells and tumorigenic HEK 293 cells but not in normal NIH3T3 fibroblast cells. Subsequently, trypan blue assay and comet assay showed CA to cause cell death and DNA damage, respectively, in the MDA-MB-231 cells. Using AO/EtBr staining and sub-G1 analysis, we further established CA to increase apoptosis. Additionally, immunoblotting showed the abundance of TNFA, TNF receptor 1 (TNFR1) and cleaved caspase-8/-3 pro-apoptotic proteins to increase with CA treatment. Subsequently, blocking of TNFA-TNFR1 signalling by small molecule inhibitor, R-7050, reduced the expression of cleaved caspase-8 and caspase-3 at the protein level.

***Conclusion*:** Thus, from the above observations, we can conclude that CA is an effective anticancer agent that can induce apoptosis in breast cancer cells *via* TNFA-TNFR1 mediated extrinsic apoptotic pathway.

Keywords: Cinnamic acid, MDA-MB-231, triple negative breast cancer, TNFA, TNFR1, R-7050, caspase, apoptosis.

1. INTRODUCTION

Breast cancer is the most common cancer in females that occurs due to uncontrolled division of the cells in the breast tissue. It has the highest incidence and is the first leading cause of cancer deaths among women worldwide (GLOBOCAN 2018). In the year 2018, it was found to be responsible for an estimated age-standardized mortality rate (ASR) of 1.3 x 10^5 individuals throughout the world. Incidence of breast cancer in India was estimated to be 1.15 x 10^5 new patients in 2018 and is projected to almost double by 2040 [1]. Triple-negative breast cancer (TNBC) accounts for around 10-20% of all the breast cancer cases [2]. These cancer cells do not express estrogen receptors (ER), progesterone receptors (PR) and HER2 receptors. They are fast growing, have limited treatment options, and have a worse prognosis (outcome) [2].

Several studies have reported that consumption of vegetables and fruits reduces the risk of malignant growth and protects against cancers [3-8]. One such anti-tumoral compound isolated from plant is cinnamic acid (CA). CA, also known as 3-phenylpropenoic acid, is an aromatic fatty acid component of cinnamon [9, 10]. CA and its derivatives can be found in cinnamon bark, coffee, cereals, fruits such as apple, citrus, berries and pear, vegetables such as potato, tomato, artichoke, beetroot and celery and propolis [9, 11, 12]. CA can also be synthesized commercially by Perkin reaction from benzaldehyde and acetic anhydride using sodium acetate as catalyst [13]. CA belongs to a class of auxin which is a plant hormone controlling cell growth and differentiation [14]. It is known to improve pancreatic β-cell functionality, cause insulin secretion, and enhance glucose uptake [15-17]. It can damage plasma membrane integrity of microorganisms and induce production of reactive oxygen species (ROS) thereby having an antimicrobial effect [18]. CA has also been used with other anti-tuberculosis drugs against Mycobacterium tuberculosis [19]. Some CA derivatives such as p-coumaric acid is used in treatment of malaria [20]. CA has been found to have cardioprotective effect in rat model of myocardial ischemic due to its antioxidative and anti-inflammatory properties [21].

Several studies have shown CA and its natural analogues to have anti-cancer properties [10, 14, 22-25]. CA was found to induce apoptosis in human nasopharyngeal cancer by upregulating FAS ligand, BAX and caspase-3 and downregulating BCL-2 [26]. CA reduced melanoma cell invasion by decreasing the expression of collagenase matrix metalloproteinase-2 (MMP-2) and increasing immunogenicity by stimulating the expression of HLA-A3 (class-I major histocompatibility antigen) [10]. Moreover, cis-CA and trans-CA were found to reduce the expression of MMP-2 and MMP-9 by modulating the expression of TIMP1, AP-1 and NF-kB in lung adenocarcinoma cells [27, 28]. Niero *et al.* showed human melanoma cells, HT-144, to undergo microtubule depolymerization on exposure to CA [29]. Trans-CA was found to increase the expression of acetylated-H3, cleaved caspase-3, cleaved PARP, BAX and reduce BCL2 expression in xenograft colon cancer model in nude mice. This antitumor activity of trans-CA was attributed to inhibi-

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